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Dissertation submitted to the University of Aveiro to meet the requirements for the Degree of Master Biotechnology, performed under the scientific guidance of Prof. Luísa Serafim, Assistant Professor at Department of Chemisty, University of Aveiro, and Dr. Simona Rossetti, Researcher at Istituto di Ricerca sulle Acque – Consiglio Nazionale delle Ricerche (IRSA/CNR).

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palavras-chave

Polihidroxialcanoatos, Culturas Mistas Microbianas, HSSL, FISH, PCR, 16S rDNA

resumo

Atualmente uma das grandes preocupações a nível mundial centra-se no desenvolvimento de novos materiais sustentáveis que possam ser usados em substituição dos produtos derivados do petróleo. Por este motivo, o estudo dos processos de produção de bioplásticos tem sido alvo de pesquisa intensiva de forma a conseguir alcançar a otimização e redução dos custos de produção destes materiais. Neste contexto, o estudo da produção de polihidroxialcanoatos (PHAs) por culturas microbianas mistas (MMC) faz todo o sentido. Neste trabalho, o processo de três passos foi testado para a produção de PHA. Numa primeira etapa, um subproduto da indústria papeleira, o licor de cozimento ao sulfito ácido (HSSL) foi submetido a fermentação acidogénica para produzir uma mistura rica em ácidos gordos voláteis (VFAs), que foi usada como substrato nas duas etapas seguintes. Na segunda etapa, a MMC recolhida numa estação de tratamento de águas residuais (WWTP) foi submetida a condições de alimentação dinâmica aeróbia (ADF) num reator descontínuo sequencial (SBR), de forma a selecionar os organismos acumuladores de PHA. Um estado pseudo-estacionário foi atingido após 60 dias de operação do SBR. Num terceiro passo, a capacidade da MMC selecionada em acumular PHA foi testada. A MMC atingiu um máximo de acumulação de PHA de 47,1%.

A comunidade bacteriana foi analisada por *fluorescence in situ hybridization* (FISH). Foram identificadas bactérias pertencentes aos seguintes grupos: *Beta-* (44,7 ± 2,7%), *Alfa-* (13,6 ± 1,3%) e *Gammaproteobacteria* (2,40 ± 1,1%) e *Bacteroidetes* (9,20 ± 3,8%).

A nível de género, dentro da classe *Betaproteobacteria*, uma quantidade considerável de *Acidovorax* (25,9 ± 3,1%) foi encontrada e foram detectadas em pequenas concentrações *Thauera* (0,72 ± 0,25%) e *Azoarcus* (0,62 ± 0,02%). *Defluvicoccus* relacionada com organismos formadores de tétradas (0,75 ± 0,53%), pertencendo à classe *Alphaproteobacteria*, foram detectadas em pequena quantidade.

Uma biblioteca de clones foi preparada e vários clones foram identificados como organismos previamente descritos como produtores de PHA.

keywords Polyhydroxyalkanoates, Mixed Microbial Culture, HSSL, Aerobic Dynamic Feeding, FISH, PCR, 16S rDNA

abstract

Currently one of the greatest concerns worldwide focuses on the development of new sustainable materials that can be used to replace petroleum-based products. For this reason, the study of the production of bioplastics has been subjected to considerable research in order to achieve process optimization and production costs reduction. In this context, the study of the production of polyhydroxyalkanoate (PHA) by mixed microbial cultures (MMC) is worthwhile.

In this work, the three-step process was tested for PHA production. In a first step, a feedstock of the paper industry, hardwood sulphite spent liquor (HSSL) was submitted to acidogenic fermentation in order to produce a mixture rich in volatile fatty acids (VFAs), which was used as substrate in the next two steps. In the second step, a MMC collected from a wastewater treatment plant (WWTP) was submitted to Aerobic Dynamic Feeding (ADF) conditions in a Sequencing Batch Reactor (SBR) in order to select PHA-accumulating organisms. A pseudo-stationary state was reached after 60 days of SBR operation. In the third step, the ability of the selected MMC to accumulate PHA was tested. The selected MMC reached a maximum PHA content of 47.1%.

The bacterial community was analysed through fluorescence *in situ* hybridization (FISH). Bacteria belonging to the four main classes were identified: *Beta*- (44.7 ± 2.7%), *Alpha*- (13.6 ± 1.3%) and *Gammaproteobacteria* (2.40 ± 1.1%) and *Bacteroidetes* (9.20 ± 3.8%).

Regarding the genus level, inside the *Betaproteobacteria,* a considerable amount of *Acidovorax* (25.9 ± 3.1%) was found and only small amounts of *Thauera* (0.72 ± 0.25%) and *Azoarcus* (0.62 ± 0.02%) were detected. *Defluvicoccus* related to Tetrad Forming Organisms (0.75 ± 0.53%) belonging to *Alphaproteobacteria* was detected in small amount.

A clone library was prepared and several clones were identified as organisms already described as PHA-producers.

Contents

Figures

Tables

Abbreviations

ADF	Aerobic dynamic feeding
AFR	Acidogenic fermentation reactor
AN/AE	Anaerobic/Aerobic process
BLAST	Basic Local Alignment Search Tool
CoA	Coenzyme A
COD	Chemical Oxygen Demand
CSTR	Continuous Stirred Tank Reactor
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dissolved Oxygen
EPS	Extracellular Polymeric Substances
F/F	Feast and Famine ratio
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein Isothiocyanate
GC	Gas Chromatography
GAOs	Glycogen-accumulating organisms
HRT	Hydraulic Retention Rime
HB	Hydroxybutyrate
HSSL	Hardwood Sulphite Spent Liquor
HV	Hydroxyvalerate
LS	Lignosulphonates
MAAS	MicroAerophilic-Aerobic System
MCL	Medium chain-length
MMC	Mixed microbial cultures

NREL	(American) National Renewable Energy Laboratory
NADPH	Nicotinamide adenine dinucleotide phosphate
OLR	Organic Loading Rate
OMEs	Olive Oil Mill Effluents
ORP	Oxidation-reduction potential
OUR	Oxygen Uptake Rate
P(3HB)	Poly(3-hydroxybutyrate)
P(3HV)	Poly(3-hydroxyvalerate)
P(3HB-*co*-3HV)	Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate)
P(3H2MB)	Poly-3-hydroxy-2-methylbutyrate
P(3H2MV)	Poly-3-hydroxy-2-methylvalerate
PAO	Polyphosphate-accumulating organisms
PCR	Polymerase Chain Reaction
PHA	Polyhydroxyalkanoate
PHAs	Polyhydroxyalkanoates
PP	Polypropylene
RT-PCR	Reverse Transcriptase-polymerase Chain Reaction
RT	Retention Time
SBR	Sequencing Batch Reactor
SCL	Short-chain-length
SRT	Sludge Retention Time
TFO	Tetrad Forming Organisms
TSS	Total Suspended Solids
VFAs	Volatile Fatty Acids

VSS	Volatile Suspended Solids
WWTP	Waste Waters Treatment Plant

1. Introduction

Plastics present versatile qualities of strength, lightness, durability and resistance to degradation. They have become an important material to enhance the comfort and quality of life. Plastics are an essential part of almost all industries and have replaced glass and paper in packaging. However, these very desirable properties have now become their greatest problem (Khanna and Srivastava 2004). The exponential growth of the human population has led to the accumulation of huge amounts of non-degradable waste materials across our planet. Living conditions in the biosphere are therefore changing dramatically, in such a way that the presence of non-biodegradable residues is affecting the potential survival of many species (Luengo et al 2003). As the conventional plastics derive from oil, other problems were raised. The limitation of oil reserves and the instability in oil prices are two of the drawbacks of the consumption of these resources (da Costa Sousa et al 2009). In addition, several environmental changes are being caused by the rise of greenhouse gases emissions (Jefferson 2006). In response to the harmful effects of plastics on the environment, there is a considerable interest in the development of biodegradable materials (Choi and Lee 1997). Among the various biodegradable polymeric materials, polyhydroxyalkanoates (PHAs) attracted industrial attention because of their potential use as practical biodegradable and biocompatible thermoplastics (Tsuge 2002).

Although PHAs are relatively well characterized in terms of properties and market applications, their price is still higher than that of synthetic plastics (Chanprateep 2010). To lower polyhydroxyalkanoate (PHA) production costs, genetic engineering technology, pathway modification or synthetic biology approaches should be engaged to develop PHA-producing strains able to grow to high cell density within a short period of time on lower cost substrates under less demanding fermentation conditions (Chen 2009).

Currently, industrial PHA production relies on pure microbial culture fermentation technology with high costs associated with carbon substrate, fermentation operation and downstream processing (Albuquerque et al 2011). In the last two decades, research has focused on the development of alternatives to substantially decrease PHA production costs (Serafim et al 2008a). The combined use of waste organic carbon and mixed microbial cultures (MMC) could significantly decrease the price of PHAs, allowing to save energy, since no sterilization is required, reducing production costs and minimizing the need for

control equipment (Johnson et al 2010).

Taking into account the emergency in developing alternatives to conventional plastics and the increasing potential in using MMCs and real substrates to produce PHAs, this research as emerged. The main objectives of this research were:

- ✓ Operation of a sequencing batch reactor (SBR) under aerobic dynamic feeding (ADF) conditions in order to select a stable PHA-accumulating culture;
- ✓ Test the ability of the selected culture to accumulate PHA through several batch tests, using as substrate the VFA-rich effluent from the acidogenic fermentation reactor (AFR);
- ✓ Analyse the evolution of microbial community composition through fluorescence *in situ* hybridization (FISH) analysis and identify the best PHA producers, as well as their relative abundances;
- ✓ Elaboration of 16S rDNA clone library and determine the presence of microorganisms known to be PHA producers.

2. State of the Art

2.1. Bioplastics

Plastics are present in our daily life in the form of disposable utensils, packaging, furniture or appliances, thereby improving our quality of life and comfort. Over the past years, the use of plastics in packaging and other products exacerbated the problem of solid waste disposal. The slow rate of degradation of these plastics results is a serious pollution problem, since they persist in the environment for an average of 100 years. For these reasons, petrochemical-based plastics are accumulated in the environment at a rate of 25 million tonnes/year (Lee et al 1991; Castilho et al 2009).

Plastic disposal is often problematic. In landfills, the degradation is very slow and incineration is expensive and generates toxic gas emissions (Reddy et al 2003; Khanna and Srivastava 2004). Recycling is difficult because it requires a preliminary screening for separation of different types of plastics and the process itself results in changes in polymer properties (Castilho et al 2009). Moreover, the presence of a wide variety of additives such as pigments, coatings, fillers, limits the use of the recycled material (Khanna and Srivastava 2004). In response to this problem and to the harmful effects of plastic wastes on the environment, there is considerable interest in the development of bio-based and biodegradable alternatives.

Biomaterials are natural products that are synthesized and/or catabolized by different organisms with a great interest from a biotechnological point of view (Luengo et al 2003). These biopolymers can be extracted directly from natural resources or chemically polymerized from organic monomers. Apart from being considered biodegradable, as various microorganisms can assimilate them, they are also biocompatible, since they do not show any toxicity to the human body (Luengo et al 2003). As such, they have several advantages over any other synthetic product (Khanna and Srivastava 2004).

2.2. PHAs

In 1888, Beijerinck was the first to observe PHAs granules in bacterial cells. A few years later, in 1926, Lemoigne described the composition of a PHAs as a homopolyester of 3-hydroxybutyric acid, to which he gave the name of poly(3-hydroxybutyrate) (P(3HB)) (Lemoigne, 1926).

PHAs are aliphatic polyesters mostly constituted by monomers of 3-

hydroxyalkanoates. They are produced under specific environmental and nutritional conditions and accumulated intracellularly as amorphous granules or inclusion bodies. The accumulated polymer allows for the survival of microorganisms in the absence of substrate, as they function as intracellular reserves of carbon and energy (Luengo et al 2003; Verlinden et al 2007; Serafim et al 2008a). PHAs are synthesized by living organisms such as bacteria, fungi or plants and more than 250 species of microorganisms are known to have this capability (Lee 1995). Bacteria are used in the large scale production of PHAs (Verlinden et al 2007). All the industrial processes so far implemented use pure microbial cultures which may accumulate PHAs up to 90% of the cell dry weight (Lee and Choi 1998; Madison and Huisman 1999).

The general chemical structure of the PHAs monomers is represented in Figure 1 and some examples of PHAs polymers are shown in Table 1. In the homopolymer P(3HB), n is equal to 1 and R is a methyl group. This is the most common and best-characterized PHA (Dias et al 2006; Castilho et al 2009). Typically, PHAs have high molecular weights, between 50,000 to 1,000,000 Da (Reddy et al 2003). PHAs can be divided into three groups in function of the number of carbons: short-chain-length PHAs (scl-PHAs) with 3 to 5 carbons, medium-chain-length PHAs (mcl-PHAs), which vary between 6 to 14 carbons and long-chain-length PHAs (lcl-PHAs), with more than 14 carbons (Singh and Mallick 2008).

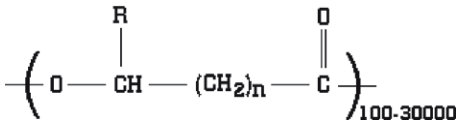


Figure 1. Chemical structure of PHAs monomers (Reddy et al 2003)

Table 1. PHAs and corresponding R-groups (Verlinden et al 2007)

R-group	Full name	Short name
CH_3	Poly(3-hydroxybutyrate)	P(3HB)
CH_2CH_3	Poly(3-hydroxyvalerate)	P(3HV)
$CH_2CH_2CH_3$	Poly(3-hydroxyhexanoate)	PHH_x

There are many types of PHAs with different monomer compositions, which influence the physical and mechanical properties of PHAs (Khanna and Srivastava 2004). P(3HB) has a low oxygen permeability and better thermoplastic properties when compared

to petroleum-based plastics, but has poor mechanical properties, since it is more rigid and brittle that polypropylene (PP) (Sudesh and Iwata 2008). It is well known that P(3HB) homopolymer and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) copolymers of low hydroxyvalerate (HV) content are stiff and brittle, with poor impact strength, because of the relatively high crystallinity of the materials. This brittleness has been a reported obstacle to the practical applications of these materials, and significant research effort has been devoted to manipulating these mechanical properties. One of the most direct solutions for improving mechanical properties of PHAs is the incorporation of high contents of HV monomer, which results in a copolymer more elastic and flexible (Serafim et al 2004; Bengtsson et al 2008b).

Compared to P(3HB), P(3HB-*co*-3HV) copolymers with higher HV contents have decreased stiffness and brittleness, increased flexibility (higher elongation to break), and increased tensile strength and toughness (Laycock et al 2013). The presence and relative proportion of each monomer depend on the type of substrate supplied. Mixtures of substrates are generally used to obtain copolymers with different monomer compositions, aiming at the tailored synthesis of PHA with given target mechanical properties (Dias et al 2006).

PHAs can be degraded under anaerobic and aerobic conditions by various microorganisms in many ecosystems such as soil, sewage, sea water or lakes (Lee 1995; Castilho et al 2009). It has been found that the rate of biodegradability of PHAs in natural environments is influenced by factors such as type of microbial population, temperature, moisture level, pH, nutrient supply as well as composition, crystallinity, additives and surface area of PHA itself (Khanna and Srivastava 2004).

Another important property of PHAs is their biocompatibility. This means that PHAs are not toxic to the human body and its degradation does not result in any toxic compound (Castilho et al 2009). This feature allows for the use of PHAs in numerous medical applications, since they are assimilated harmlessly by the human body (Verlinden et al 2007).

There are several types of PHAs, such as Biopol^{TM}, Biomer^{TM}, Nodax^{TM} and Biocycle^{TM}. These polymers are produced by natural pure cultures of *Alcaligenes latus* (Biomer^{TM}) and *Bacillus spp*. (Biocycles^{TM}) or by genetically modified strains of *Escherichia coli* (Biopol^{TM}) or *Cupriavidus necator* (Nodax^{TM}) (Singh and Mallick 2008;

Chen 2009).

The application of PHAs in commodities depends on three core mechanical properties: elongation at break, which is a measure of toughness or total deformation before fracture; Young's modulus which is a measure of stiffness; and ultimate tensile strength, which is a measure of strength prior to the onset of permanent plastic deformation (Laycock et al 2013). Table 2 summarizes different properties of several types of PHAs.

Table 2. Properties of commercial PHAs (Laycock et al 2013)

	P(3HB)		**P(3HB) copolymers**		**P(3HB-*co*-3HV)**			**P(3HB-*co*-3HHx)**
	Biomer 240 **Injection mold**	**Biomer P226** Injection mold	**Mirel P1001** Injection mold	**Mirrel P1002** Extrusion and injection	**ENMAT** Injection mold	**Biocycle 1000** Extrusion and injection	**Biocycle 2400-4** Extrusion, injection and fibre	**Kaneka** Foam mold
Melt flow rate (g/10 min)	5-7	9-13				10-12	15-25	5-10
Density (g/cm^3)	1.17	1.25	1.39	1.3	1.25	1.22	1.2	1.2
Crystallinity (%)	60-70	60-70				50-60		
Tensile strength (MPa)	18-20	24-27	28	26	36	30-40	25-30	10-20
Elongation (%)	10-17	6-9	6	13	5-10	2.5-6	20-30	10-100
Flexural strength (MPa)	17	35	46	35	61			
Flexural modulus (GPa)			3.2	1.9	1.4			
Melting temperature (°C)					147	170-175		0.8-1.8
VICAT softening point (°C)	53	96	148	137	143			120-125

2.3. Applications of PHAs

Like the petrochemical-based synthetic plastics, most PHAs are thermoplastics and can be thermally processed using existing technologies in the plastic industry. The properties of PHAs can also be tailored to suit numerous applications ranging from stiff packaging goods to highly elastic materials for coatings (Sudesh and Iwata 2008). PHAs applications can be divided into three areas: industrial, agricultural and medical (Philip et al 2007). The use of PHAs in industry has been gaining territory (Sudesh and Iwata 2008). Initially, PHAs were used to make everyday articles such as shampoo bottles and packaging materials. In recent times, other applications were added, such as medical implants, drug delivery carriers, printing and photographic materials, fine chemicals and nutritional supplements (Chen 2009). Due to their piezoelectric nature, it is also possible

to use PHAs to make pressure sensors, stretch and acceleration measuring instruments, shock wave sensors and lighters. The gas barrier property of P(3HB-*co*-3HV) is useful for applications in food packaging and for making plastic beverage bottles (Philip et al 2007).

In agriculture, PHAs are used in the production of devices for controlled release of fertilizers, herbicides and insecticides (Philip et al 2007). They can also be used as bacterial inoculants used to enhance nitrogen fixation in plants (Kadouri et al 2005), encapsulation of seeds, biodegradable films for crop protection or in the construction of greenhouses (Verlinden et al 2007).

Due to their properties of biodegradability and biocompatibility, several medical applications were already developed. PHAs can be turned into drug controlled release matrices. PHAs monomers, especially hydroxybutyrate (HB), were found to have therapeutic effects on Alzheimer and Parkinson, osteoporosis and even memory improvement (Chen 2009). PHAs are used in tissue engineering, artificial organ construction, surgical sutures and surgical glue (Philip et al 2007; Wu et al 2009).

The primary obstacle for the replacement of synthetic plastics by biopolymers is their significant cost difference; the P(3HB) cost ranges from 2.5–3.0 €/kg (Chanprateep 2010) up to 12 €/kg (Castilho et al 2009), and the copolymer P(3HB-*co*-3HV) cost can also range from 3.0–5.0 €/kg (Chanprateep 2010) to 12 €/kg (Castilho et al 2009), compared with a cost of less than 1 €/kg (Chanprateep 2010) for conventional petroleum-based polymers.

2.4. PHAs production

2.4.1. Producing microorganisms

The selection of a microorganism for the industrial production of PHAs should rely on several factors including the cells' ability to utilize inexpensive carbon sources, growth and polymer synthesis rates, and maximum polymer storage content. Recovery of PHAs should also be considered because it contributes significantly to the production costs (Khanna and Srivastava 2004).

Over 150 different types of hydroxyalkanoate monomers have been identified, being synthesized by over 300 species (Reis et al 2011). The industrial production of PHAs is carried out by natural or recombinant pure cultures of microorganisms such as *Escherichia coli, Alcaligenes latus, Bacillus spp., Cupriavidus necator, Aeromonas*

hydrophila, Pseudomonas putida and Pseudomonas oleovorans. The use of recombinant strains allows to achieve higher yields with lower costs (Lemos et al 2006). However, the use of pure cultures still has several disadvantages that can be overcome through the use of MMCs (Serafim et al 2008a). Several strains are commonly used for pilot and large-scale production of PHAs and the results obtained with these are resumed in Table 3.

Table 3. Wild type and industrial bacteria strains commonly used for pilot and large scale PHAs production (Chen 2009)

Strain	DNA manipulation	Final Cell Dry Weight (CDW) (g L^{-1})	Final PHA content (% CDW)	Company
Cupriavidus necator	No	> 200	> 80%	Tianjin North. Food, China
Alcaligenes latus	No	> 60	> 75%	Chemie Linz, btF, Austria
Escherichia coli	phbCAB + vgb	> 150	> 80%	Biomers, Germany Jiang Su Nan Tian, China
Cupriavidus necator	No	> 160	> 75%	ICI, UK Zhejiang Tian An, China
Cupriavidus necator	No	> 100	> 75%	Metabolix, USA
Escherichia coli	phbCAB			Tianjin Green Biosci. China
Cupriavidus necator	$phaC_{Ac}$	> 100	> 80%	P&G, Kaneka, Japan
Aeromonas hydrophila	No	< 50	< 50%	P&G, Jiangmen Biotech Ctr, China
Aeromonas hydrophila	phbAB + vgb	~ 50	> 50%	Shandong Lukang,
Pseudomonas putida	No	~ 45	> 60%	ETH, Switzerland
Bacillus spp.	No	> 90	> 50%	Biocycles, Brazil

2.4.2. Pure cultures and Mixed Microbial Cultures

MMCs have emerged as an alternative to pure cultures since operating costs can be reduced by 50%. The use of MMCs combined with the reuse of industrial or agricultural by-products can reduce operating costs in 85% (Tsuge 2002). A variety of low cost carbon substrates (e.g., starch, tapioca hydrolysate, whey, and molasses) have been tested for PHA production by pure cultures, but PHA production costs remain very high, mainly due to investment and operating costs (Albuquerque et al 2007). The utilization of MMCs facilitates the use of complex substrates since microbial population can adapt continuously

to changes in substrate. Moreover, there is no need for sterilization and sterile fermentation systems, which contributes to the reduction of the final PHA price (Serafim et al 2004). The process also reduces costs, since less sophisticated equipment is used and less control is needed (Lee 1995). MMCs do not have a defined composition and the selection process gradually enriches the culture in organisms of interest, by imposing certain operation conditions. The MMC selected for production of PHAs has a high PHA storage capacity due to the operating conditions, which limits its primary metabolism (Dias et al 2006).

The MMCs from Wastewater Treatment Plants (WWTP) have the ability to produce PHAs as storage of carbon and energy under transient conditions of availability of substrate. The MMCs have the ability to rapidly store the substrate when it is available, converting it into PHA, which is consumed during famine phase (Serafim et al 2008a; Coats et al 2010). In addition, MMCs have the ability to adapt to substrates whose composition is unknown, allowing for the use of cheap substrates such as industrial wastes (Serafim et al 2008a).

Johnson et al. (2009) was able to maintain SBRs for PHA producing cultures enrichment running continuously for over 4 years under various conditions without stability problems (Johnson et al 2009). Serafim et al. (2008b) also reported a stable reactor performance of a PHA producing SBR for over 2 years (Serafim et al 2008b). While the risk of contamination is a big problem for pure culture processes, the use of an open system for mixed culture PHA production actively supports the introduction of new organisms into the reactor that could potentially be more competitive PHA producers than the established community (Johnson et al 2009).

In spite of the many efforts devoted in the last decades to improve this process, the volumetric productivities and contents are still lower and than those achieved for pure cultures. Although the specific productivities are higher in MMC processes, cell concentrations are below the values required to reach the volumetric productivities of the pure culture processes (Serafim et al 2008a). Johnson et al. (2009) were able to select a virtually pure culture highly enriched in PHA-accumulating organisms and demonstrating a very high PHA storage capacity (up to 89%) by appropriately manipulating the operating conditions imposed on an open SBR fed with acetate (Johnson et al 2009).

2.4.3. Metabolism for PHA synthesis by MMCs

The metabolism for PHAs synthesis is well defined for pure cultures, but only a few metabolic studies involving MMCs were reported (Lemos et al 2006). As such, it is assumed that the PHA production metabolism in MMCs is similar to the one described for pure cultures (Dias et al. 2006).

The feedstock used in PHAs production is normally enriched with volatile fatty acids (VFAs) and/or sugar-based compounds (Serafim et al 2008a). Acetate is the most widely used substrate in the ADF process and it is preferably stored as P(3HB) (Serafim et al 2004). Figure 3 shows three metabolic pathways involved in the synthesis of PHAs.

Firstly, the organic acids molecules are transported across the cell membrane and activated to the corresponding acyl-coenzyme A (CoA) or, for glucose, breakdown through glycolysis till acetyl-CoA (Serafim et al 2008a). There are three known possible pathways for the production of PHAs that vary according to the substrate used. For acetate, two molecules of acetyl-CoA condense to acetoacetyl-CoA by β-ketothiolase. This product is reduced (by acetoacetyl-CoA reductase) to 3- hydroxybutyryl-CoA at the expense of conversion of reduced NADPH to $NADP^+$. This monomer is the direct precursor for the synthesis of P(3HB) by PHB synthase (Serafim et al 2008a).

On the other hand, when propionate is the substrate, three different precursors can be produced. If two molecules of propionyl-CoA condense, they will originate 3-hydroxy-2-methylvaleryl-CoA, the precursor of poly-3-hydroxy-2-methylvalerate (P(3H2MV)). If acetyl-CoA is present or if is formed through the breakdown of propionyl-CoA, the addition of acetyl-CoA and propionyl may form poly-3-hydroxyvalerate (P(3HV)) or poly-3-hydroxy-2-methylbutyrate (P(3H2MB)) (Serafim et al 2008a). The propionate is catalysed principally for the production of P(3HV), although some 3HB production can occur due to formation of its precursor, 3-hydroxybutyryl-CoA, by the condensation of two molecules of acetyl-CoA (Lemos et al 2006). Butyrate and valerate can be used for the direct production of P(3HB) or P(3HV) (Serafim et al 2008a).

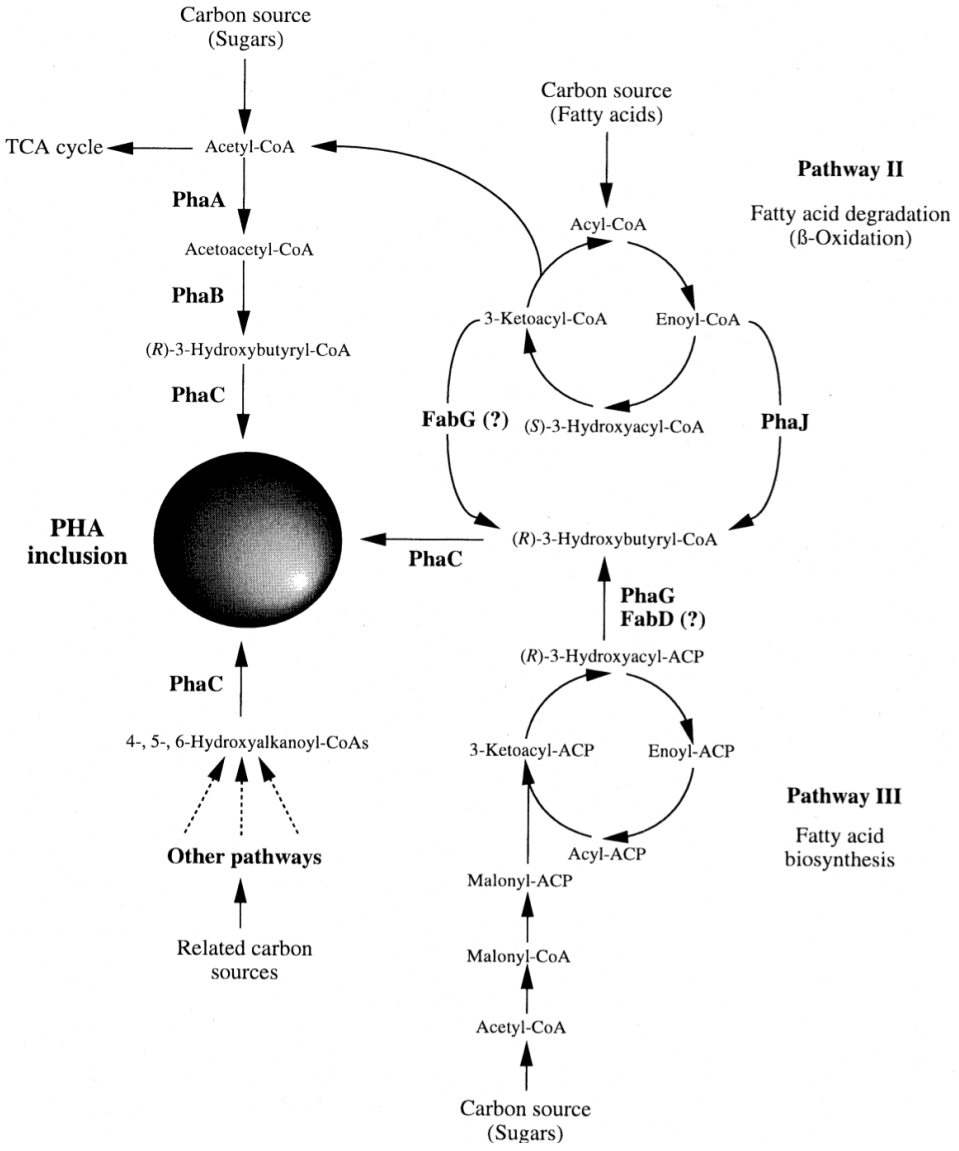


Figure 2. Metabolic pathways for production of hydroxyalkanoate monomers for PHA synthesis (Sudesh et al 2000)

2.5. Three-step process

Dionisi et al. (2005c) developed a three-stage process for PHA production from organic wastes. During the first stage of acidogenic fermentation, a high-concentration biodegradable waste is transformed into a mixture of acetate and other carboxylic acids. In the second stage, a SBR is operated with periodic feeding in order to enrich the MMC in bacteria with a high storage response to be used in the third stage. The final stage is operated in batch, with an excess of external substrate in order to increase the MMC PHA content (Dionisi et al 2005c). Figure 3 shows a schematic representation of the three-step process.

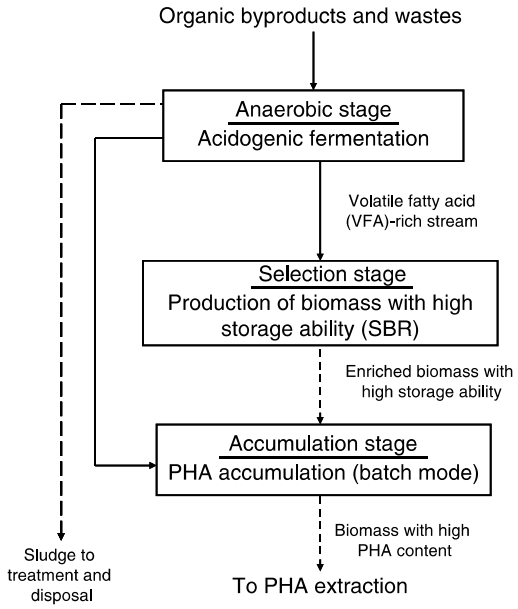


Figure 3. Schematic representation of a three-stage process for PHA production from waste/surplus-based feedstocks (Reis et al 2011)

2.5.1. Acidogenic fermentation

PHA production costs can be reduced if waste organic carbon sources are used as substrates. Many waste streams from agriculture and industry like cane and beet molasses, cheese whey, plant oils and hydrolysates of starch, cellulose and hemicellulose, are potentially useful substrates. When an open-culture system is used, not all substrates are equally suitable. For example, starch and cellulose hydrolysates could lead to the growth of glycogen-accumulating organisms. An alternative to overpass this problem is the anaerobic digestion of the organic substrates, where soluble organic compounds are fermented into organic acids, such as acetic, propionic, butyric, and lactic acid, and other fermentation products, such as alcohols and hydrogen. This mixture of VFAs is readily converted to PHA (Carta et al 2001; Reis et al 2003; Salehizadeh and Van Loosdrecht 2004).

VFAs derived from waste feedstocks have important applications in addiction to serve as substrate for PHA production. Microbial production of organic acids is a promising approach for obtaining building-blocks from renewable carbon sources and VFAs are extremely useful as starting materials for the chemical industry (Sauer et al 2008).

The acidogenic fermentation is an important step in anaerobic digestion of organic compounds to methane and carbon dioxide. In this process, microbial species ferment

dissolved organic compounds into organic acids and other fermentation products, such as alcohols, under anaerobic conditions. Since the produced VFAs are also substrates for PHA production, acidogenic fermentation can serve as a suitable pre-treatment in the process of PHA production (Bengtsson et al 2008a).

The composition of the produced PHA depends on the types of VFAs that are used as substrate. Acetate and butyrate have a tendency to form 3HB monomers whereas presence of propionate tends to increase the amount of HV in the polymer (Dias et al 2006; Castilho et al 2009). The composition of VFAs produced under acidogenic fermentation can be affected by conditions such as pH, retention time (RT) and temperature (Albuquerque et al 2010b). So, manipulating operating conditions of the anaerobic fermentation step can be used to control the biopolymer composition in the PHA production step (Albuquerque et al 2011).

Almost all the studies describing PHA production by MMCs perform culture selection and PHA accumulation in two separate steps (Dionisi et al 2003; Serafim et al 2004; Dias et al 2006). Only few works report the use of a three-step production process, in which an anaerobic fermentation step precedes the culture selection and polymer accumulation steps. Bengtsson et al. (2008b) developed a three-step process for the production of PHA from paper mill effluents, including the continuous fermentation of the paper mill effluent followed by culture selection and PHA production using the fermented effluent as substrate (Bengtsson et al 2008b). Albuquerque and co-workers (2007) developed a three-stage process to produce PHA from sugar cane molasses. The process includes molasses acidogenic fermentation, selection of a PHA-accumulating culture and PHA batch accumulation using the enriched sludge and fermented molasses (Albuquerque et al 2007).

Different feedstocks were already investigated for acidogenic fermentation toward PHA production. These feedstocks include cheese whey, pulp and paper mill effluents (Bengtsson et al 2008a), sugarcane molasses (Albuquerque et al 2007) and olive oil mill effluents (OMEs) (Beccari et al 2009). Cheese whey, paper mill effluents, and sugarcane molasses were all found to be readily and almost fully fermentable, producing mainly VFAs (acetate, propionate, butyrate, and valerate) as fermentation products (Albuquerque et al 2007; Bengtsson et al 2008a). OMEs were fermented with a lower yield and the fermented stream contained both VFAs (acetic, propionic, and butyric acid) and alcohols

(methanol, ethanol, and butanol) accounting for 32% and 22% of the overall COD, respectively (Beccari et al 2009).

2.5.2. Culture selection

The selection of a microbial population with a high storage capacity is one of the main challenges in MMC processes (Serafim et al 2008a). If the selected population is very heterogeneous in terms of the storage capacity, that may have a negative impact in the downstream process, since it would result in the reduction of average PHA cell content and increase of PHA extraction costs. Thus, the operation of the selection reactor should be optimized to obtain a homogeneous population with a relatively high and stable storage capacity rather than to maximize the PHA cell content (Serafim et al 2008a). The most important processes for culture selection are the Anaerobic/Aerobic process (AN/AE), the MicroAerophilic-Aerobic System (MAAS) and ADF.

Under AN/AE conditions, there are two main groups of bacteria responsible for PHA accumulation, which can be selected: polyphosphate accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs). In anaerobic conditions, these microorganisms use the carbon source for the production of PHA with simultaneous consumption of glycogen. Under aerobic conditions, after the exhaustion of the external substrate, the stored PHA is consumed for growth, maintenance and replenishment of glycogen reserves (Serafim et al 2008a). According to Dai et al. (2007), in comparison with PAOs, GAOs are more robust, reach higher PHA contents and easily produce copolymers of P(3HB-*co*-3HV) from simple substrates (Dai et al 2007). Real substrates as fermented molasses (Pisco et al 2009), fermented wastewater (Coats et al 2007) and pre-fermented paper mill effluent (Bengtsson et al 2008b), were already tested in this kind of process and the obtained PHA accumulations were of 37%, 10-25% and 48% (w/w), respectively.

Satoh et al. (1998) introduced the MAAS where a limited amount of oxygen is supplied during the anaerobic period of anaerobic–aerobic operations (Satoh et al 1998). In such conditions, microorganisms can take up organic substrates by obtaining energy through oxidative degradation of some part of the organic substrates. If the supply of oxygen is sufficient, the microorganism may be able to get enough energy for assimilative activities such as the production of protein, glycogen and other cellular components simultaneously with taking up organic substrates. However, if the supply of oxygen is

adequately controlled, the assimilative activity will be suppressed while letting the microorganism accumulate PHA (Salehizadeh and Van Loosdrecht 2004).

Among the different processes described for industrial PHA production by MMCs, the feast and famine process or ADF is the most promising because of the high sludge PHA content and productivity (Reis et al 2003). In this process, MMCs are subjected to alternating periods of presence (feast) and absence (famine) of substrate, which leads to an uneven growth of the MMC. The operation of the system with alternating phases of excess carbon followed by substrate exhaustion imposes a selective pressure. During the famine phase, the absence of carbon causes the decrease of cellular components essential for growth, such as RNA and enzymes. After the starvation period, when an excess of carbon is supplied, the PHA storage is favoured, due to the low availability of material essential for growth (Majone et al 1996). In the absence of substrate, only organisms able to store PHA will survive, since the stored polymer will be used as a source of carbon and energy to ensure growth and cell maintenance during famine phase (Dionisi et al 2003; Serafim et al 2004; Dias et al 2006). The ability to store internal reserves gives these microorganisms a competitive advantage over those without this ability, when facing transient substrate supply (Salehizadeh and Van Loosdrecht 2004).

In this process, the reactor is operated under intermittent substrate feed in order to favour the polymer storage capacity. The substrate is fed during a short period of time, followed by a longer period of substrate absence. This period of famine is needed to stimulate the PHA storage capacity of cells (Reis et al 2003). Figure 4 represents a possible metabolic pathway for acetate consumption under feast/famine conditions suggested by Reis and co-workers (2003).

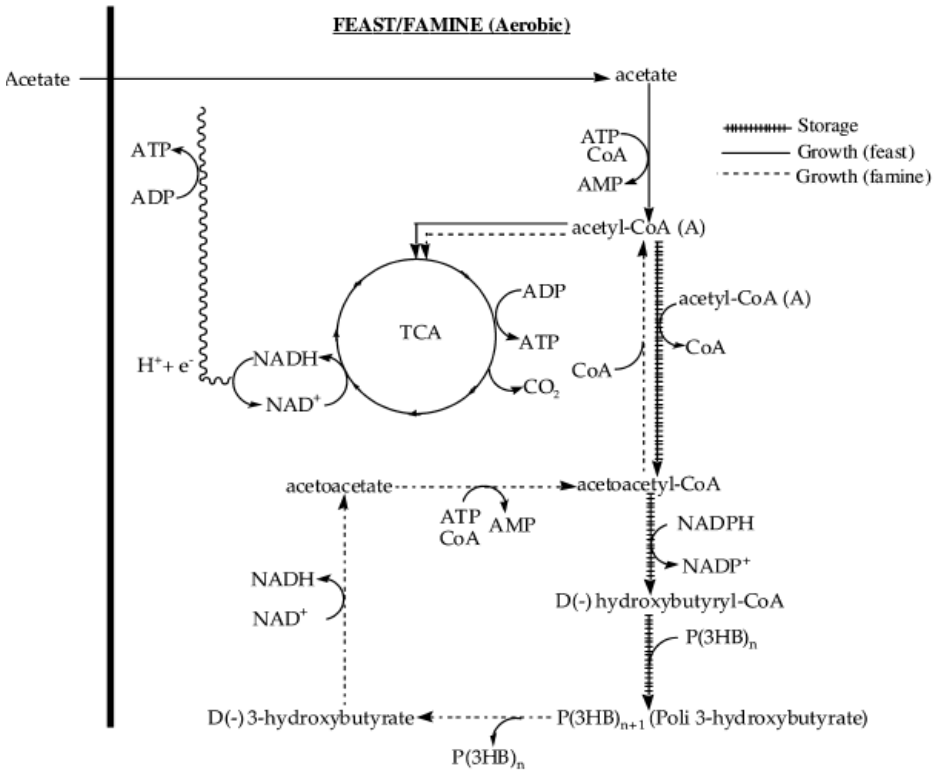


Figure 4. Possible metabolic pathway for acetate consumption under feast/famine conditions (Reis et al 2003)

The ratio between the length of the feast and famine phase (F/F ratio) is a crucial parameter affecting the performance of the selection stage (Albuquerque et al 2011). In general, low F/F ratio ensures the selection of microorganisms that are most able to store PHA and their physiological adaptation toward PHA synthesis in the feast phase (Villano et al 2014).

2.5.2.1. Microbial community characterization

During the process of selection of a PHA-accumulating culture, the microbial community should be evaluated periodically in order to monitor its evolution, identify the best producers and determine their relative abundance. The microbial characterization must be correlated with the operational conditions allowing for the enhancement of those that favour the selection of PHA-accumulating microorganisms (Serafim et al 2008a). Moreover, monitoring the culture evolution throughout the operational period allows to understand some changes in the performance of the MMC that could eventually occur (Queirós et al 2014).

Several studies focused on the microbial community structure of MMCs in PHA-producing processes have revealed the presence of a wide phylogenetic diversity, although *Thauera, Azoarcus* and *Paracoccus* have often been associated as PHA producing organisms (Dionisi et al 2005a; Dionisi et al 2005c; Serafim et al 2006; Lemos et al 2008; Moita and Lemos 2012; Queirós et al 2014; Dai et al 2015).

Dionisi et al. (2005a) used Denaturing Gradient Gel Electrophoresis (DGGE) and 16S rRNA clone library to follow the evolution of a PHA-producing MMC. The main phylogenetic groups identified were: *Betaproteobacteria, Alphaproteobacteria* and *Gammaproteobacteria* (Dionisi et al 2005a).

Dionisi et al. (2005c) used DGGE and elaborated a 16S rRNA clone library to identify PHA-accumulating organisms in an ADF reactor. The dominant group was *Betaproteobacteria* (*Thauera, Alcaligenes, Comamonas, Achromobacter*). *Gammaproteobacteria* (*Kluyvera, Pseudomonas, Acinetobacter*) and *Alphaproteobacteria* (*Xantobacter, Curtobacterium*) were also present. *Thauera* was the dominant genus and was described for the first time as a PHA-producer (Dionisi et al 2005c).

Serafim et al. (2006) used FISH and Nile Blue staining to characterize the microbial populations in two reactors operated under ADF conditions, one consuming acetate and the other propionate. *Betaproteobacteria* and *Alphaproteobacteria* were found to be the main phylogenetic groups and *Azoarcus* was identified as the genus mostly responsible for PHA-accumulation (Serafim et al 2006).

Lemos et al. (2008) used the sorting/reverse transcriptase–polymerase chain reaction (RT-PCR) approach to reveal the main composition of the PHA-accumulating biomass present in two systems fed with different substrates: acetate and propionate. Using FISH, the presence of microorganisms identified by RT-PCR was confirmed. FISH analysis showed that bacteria belonging to the genera *Amaricoccus, Thauera* and *Azoarcus* were always present in both reactors. However, clear differences in the abundance of these genera were observed between the two reactors, which could be related with the kind of carbon source utilized (Lemos et al 2008).

Moita and Lemos (2012) used FISH to identify PHA-accumulating organisms selected under ADF conditions. The majority of the identified organisms belonged to *Alphaproteobacteria*, *Betaproteobacteria*, or *Gammaproteobacteria* classes (Moita and Lemos 2012).

Queirós et al. (2014) selected a PHA-accumulating culture from a MMC and characterized the selected culture using FISH. The bacterial community was mostly constituted by *Alphaproteobacteria*, followed by *Betaproteobacteria* and *Gammaproteobacteria*. Within *Alphaproteobacteria*, small amounts of *Paracoccus* and *Defluvicoccus* related to Tetrad Forming Organisms (TFO) were detected (Queirós et al 2014).

Dai et al. (2015) investigated the potential of using wood hydrolysates as substrate to produce P(3HB) using a MMC. 16S rRNA sequencing with Illumina system was used to analyse the microbial community. *Actinobacteria*, *Alpha-* and *Betaproteobacteria* were found to be the dominant groups in the bioreactors. Several PHB-storing microorganisms belonged to *Alpha-* and *Betaproteobacteria* groups (Dai et al 2015).

In the present work, the microbial community composition is going to be identified and monitored through FISH analysis. Throughout the SBR operational period, several samples were collected and fixed in paraformaldehyde 4% in order to be preserved until submitted to the molecular analyses. A 16S rDNA clone library was also prepared.

2.5.2.2. Operational parameters

The production of PHA by MMCs subjected to a feast and famine selection strategy is usually carried out in SBRs, operated in cycles of feeding, reaction, settling and drawn (Dias et al 2006). SBRs are ideal reactors for the selection of robust populations with high ability of PHA storage, because biomass grows under transient conditions. Furthermore, this reactor is easy to control and highly flexible, allowing the rapid change of the process conditions, for example the duration of feeding and cycle length (Reis et al 2003).

The MMC response is influenced by different factors such as reactor operating conditions (sludge retention time (SRT), hydraulic retention time (HRT), pH, temperature, cycle length, organic loading rate (OLR), influent substrate concentration and nutrient concentration) as well as the feedstock (Reis et al 2011).

During the reactor operational time, several cycles were monitored in order to study the culture evolution. In these cycles, parameters such as temperature, pH and dissolved oxygen (DO) concentration were monitored.

The pH was measured in order to check its influence in PHA accumulation. Ideally, it should be between 8 – 9, since its reduction to values between 6 – 7 causes the decrease

of cellular PHA content (Dias et al 2006). Villano et al. (2010) also observed that the rates and yields of PHA storage decreased, as pH increased from 7.5 to 9.5 (Villano et al 2010).

According to Third at al (2003), limiting oxygen concentrations allows to achieve higher yields of PHA storage, since the higher the concentration of DO, the greater will be the proportion of substrate which will be used for cell growth and, consequently, less substrate will be converted to PHA (Third et al 2002).

The accumulation of storage polymers is strongly dependent on temperature and at higher temperatures (35ºC), less PHB production occurs. Nevertheless, this could be an advantage because the use of low temperatures allows for a less costly process (Krishna and Van Loosdrecht 1999). Johnson at al. (2010) concluded that at higher temperatures (30ºC) PHB storage was the dominant process in the feast phase. At lower temperatures (15ºC) the feast phase was longer and growth occurred predominantly directly on acetate rather than on stored P(3HB). This culture had a low PHB storage capacity (Johnson et al 2010).

One of the objectives of the selection stage is to produce a PHA-storing culture with the highest productivity possible, that is, the amount of biomass produced per unit of volume of reactor and per unit of time. To achieve high biomass volumetric productivities, the SBR needs to be operated at OLRs as high as possible, which corresponds to high influent substrate concentration and/or short HRT (Villano et al 2014).

Beun et al. (2002) concluded that for SRTs higher than 2 days, the yield of P(3HB) from acetate under excess nutrients was constant and, thus, independent of the specific growth rate. At a SRT below 2 days, the P(3HB) storage yield and productivity decreased sharply with the decrease of the SRT (Beun et al 2002).

Dionisi et el. (2007) studied the effect of the cycle length on the enrichment and selection of mixed cultures in SBRs. In the perspective of PHA production from organic wastes, at fixed OLR (20 gCOD L^{-1} day^{-1}) the length of the cycle played a relevant role in determining the length of the feast and famine phases. With low or high lengths of cycle, storage rates and yields were very low and the dynamic response of the biomass to substrate excess was dominated by growth. The selection of microorganisms with storage or growth response is correlated with the ratio of the feast phase/length of the cycle: storage response was observed only when the feast phase was not longer than 20% of the length of the cycle (Dionisi et al 2007).

Regarding the F/F ratio, low F/F values ensure physiological adaptation of the microorganisms, favouring PHA storage in the feast phase. High F/F ratios that result from either higher OLR/influent substrate concentration or shorter cycles can cause a partial or complete loss of the physiological adaptation, which will cause the substrate uptake to be driven predominantly toward growth (Albuquerque et al 2011).

Valentino et al. (2014) studied the effect of feed frequency on PHA production, while maintaining the same OLR, HRT and feed concentration (8.5 g COD L^{-1} of VFAs). The effect of feeding time was evaluated with a cycle length of 8 h and an increase in the storage response was observed by increasing the rate at which the substrate was fed into the reactor. By decreasing the cycle length from 8 h to 6 h and then to 2 h, changing the feed frequency or changing the organic load given per cycle, the length of the feast phase decreased from 26 to 20 and then to 19.7% of the overall cycle length, respectively, due to an increase in the substrate removal rate (Valentino et al 2014).

2.6. PHAs production from low-value feedstocks

As already mentioned, the substrate cost is one of the major contributors to the high price of PHAs, since the feedstock is estimated to account for 40% of the total production cost (Salehizadeh and Van Loosdrecht 2004). Therefore, to lower the production costs is essential to select cheaper substrates that can be used for synthesizing PHA with high yields (Lemos et al 2006).

Preliminary results of several investigations showed the possibility of using agro-industrial waste streams instead of synthetic substrates for PHA production (Dionisi et al 2005b; Albuquerque et al 2007; Coats et al 2007; Bengtsson et al 2008b; Albuquerque et al 2010b; Albuquerque et al 2011; Jiang et al 2012). Nevertheless, the PHA storage capacity obtained from those researches was still significantly lower than those of microbial enrichments selected on synthetic feedstocks. Most of these studies reported PHA content around 55% of the dry weight (Dionisi et al 2005b; Bengtsson et al 2008b; Albuquerque et al 2011). The microbial enrichment obtained by Jiang et al. (2012) could accumulate a maximum of 77% PHA of cell dry weight within 5 hours (Jiang et al 2012).

The use of MMCs as PHA-producers was increasingly investigated as a mean of reducing production costs together with the use of several surplus-based feedstocks, including: fermented molasses (Albuquerque et al 2007; Albuquerque et al 2010b; Albuquerque et al 2011), fermented paper mill effluents (Bengtsson et al 2008b; Jiang et al

2012), fermented OMEs (Dionisi et al 2005b; Beccari et al 2009), glycerol (Dobroth et al 2011; Moralejo-Gárate et al 2011), bio-oil (Moita and Lemos 2012), municipal wastewaters (Coats et al 2007) and Hardwood Sulphite Spent Liquor (HSSL) (Queirós et al 2014).

Dionisi et al. (2005) examined the production of PHA from OMEs using a three-step strategy. In the fermentation step, OMEs were anaerobically fermented to obtain VFAs. The PHA production stage was investigated through batch tests performed by using a MMC enriched in a SBR fed with a mixture of organic acids. The PHA production specific rate obtained was 420 mg COD g COD^{-1} h^{-1} (Dionisi et al 2005b).

Albuquerque et al. (2007) used a three-stage process to produce PHAs from sugar cane molasses. The three-step process used, comprised continuous acidogenic fermentation of molasses, selection of a PHA-accumulating culture under feast and famine conditions and batch PHA accumulation using the selected culture and the fermented molasses. Two different strategies were used: conducting culture selection with a synthetic substrate (acetate) and accumulation with fermented molasses or using the fermented molasses as feedstock in both steps. The effect of organic acids distribution on polymer composition and yield was evaluated with the acetate selected culture and the storage yields varied from 0.37 to 0.50 Cmmol HA/Cmmol VFA. Low ammonia concentration (0.1 Nmmol/l) in the fermented molasses stimulated PHA storage and the storage yield obtained was 0.62 Cmmol PHA/Cmmol VFA (Albuquerque et al 2007).

Bengtsson et al. (2008b) investigated the production of PHA from a paper mill wastewater using a three-stage process consisting in acidogenic fermentation to convert wastewater organic matter to VFAs, an activated sludge system operated under feast/famine conditions for enrichment in PHA-producing organisms and PHA accumulation in batch experiments. The maximum PHA content achieved was 48% of the sludge dry weigh (Bengtsson et al 2008b).

Coats et al. (2007) investigated PHA production with a MMC through an activated sludge process using carbon present in municipal wastewaters. Their goal was to demonstrate that commercial PHA production could be integrated into various configurations of municipal wastewater treatment schemes. A PHA accumulation of 53% PHA (w/w) was obtained. The obtained results suggested that the commercial production of PHA could feasibly be integrated into wastewater treatment systems (Coats et al 2007).

Beccari and co-workers (2009) investigated the performance of a three-stage process for PHA production from OMEs. In the first anaerobic stage, OMEs were fermented to VFAs in a packed bed biofilm reactor. This VFA-rich effluent was fed to the second stage, operated in an aerobic SBR, to select a MMC able to store PHA. Finally, the storage response of the selected consortia was exploited in the third aerobic stage, operated in batch conditions. The maximum value of PHA storage rate was 146 mgCOD $gCOD^{-1}$ h^{-1} (Beccari et al 2009).

Albuquerque et al. (2010a) used a 2-stage continuous stirred tank reactor (CSTR) system to carry out culture enrichment in a 3-stage process using sugar molasses as feedstock in order to determine the impact of the reactor operation mode on PHA-accumulating culture selection under feast and famine conditions. The effect of different operating parameters such as influent substrate concentration (60–120 Cmmol VFA/L) and ratio between the HRT of the two CSTRs (0.2–0.5 h/h) on the selection efficiency were assessed. The culture reached a maximum PHA content of 61% (Albuquerque et al 2010a).

Moralejo-Gárate and co-workers (2011) studied the production of PHA by a MMC using glycerol as substrate. The PHA-producing MMC was enriched using feast/famine regime in a glycerol-fed SBR. In a subsequent fed-batch PHA production step, the enriched mixed community produced PHA up to a dry weight content of 80 wt% (Moralejo-Gárate et al 2011).

Albuquerque et al. (2011) studied the possibility to control polymer composition and properties in mixed culture PHA production from fermented molasses. The effects of substrate VFA composition and feeding regime on polymer composition and structure were assessed. Continuous feeding strategy could be used to increase the HV content by 8–9% relatively to that obtained from the same feedstock using pulse feeding. Therefore, the feeding strategy could be used to manipulate polymer composition and properties. P(3HB-*co*-3HV) copolymers with HV fraction ranging from 15 to 39% were obtained, which subsequently resulted in different polymer properties. It was found that the use of a continuous feeding strategy rather than a pulse feeding strategy can allow higher rates of substrate uptake and polymer storage, originating a considerable increase of volumetric productivity. Different substrates were tested in order to study the effect of substrate VFA profile on polymer composition, yield and maximum PHA accumulation. Simulated fermented molasses with different VFA profiles and real fermented molasses were tested.

In terms of PHA storage efficiency, maximum PHA contents of 56 and 66% were obtained, with fermented molasses feed and simulated feedstock, respectively. The lower maximum PHA content relates to the slightly lower rates of substrate uptake and PHA storage observed with the real feedstock. In all the cases, P(3HB-*co*-3HV) copolymers were produced and the polymer composition was not significantly affected by using real fermented molasses instead of the simulated fermented molasses. This indicates that the molasses matrix does not introduce significant variation on the polymer composition (Albuquerque et al 2011).

Dobroth et al. (2011) investigated the potential of PHA production on crude glycerol using MMC and determined that the enriched MMC produced exclusively P(3HB) utilizing the methanol present in crude glycerol. The maximum PHA content achieved was 67% of cell dry weight (Dobroth et al 2011).

Jiang et al. (2012) used a three-step process to increase the maximum content that can be stored by a MMC using paper mill effluent as feedstock. In this work, the paper mill waste stream was firstly acidified to VFAs in a batch experiment. Then, the effluent from the acidogenic reactors was used as substrate for the selection SBR, and for the accumulation reactor operated in a fed-batch mode. The selected culture could accumulate maximum up to 77% PHA of cell dry weight within 5 hours (Jiang et al 2012).

Moita and Lemos (2012) used bio-oil resulting from the fast-pyrolysis of chicken beds as substrate to select a MMC able to produce PHA under feast/famine conditions. In this study a maximum PHA content of 9.2% (g/g cell dry weight) was achieved in a SBR operated for culture selection (Moita and Lemos 2012).

HSSL was firstly used as substrate for PHA production by MMCs by Queirós et al. (2014). The MMC selection process was performed in a SBR operated under ADF conditions and the selected PHA-accumulating culture reached a maximum PHA content of 67.6% (Queirós et al 2014).

These results demonstrate the high potential of MMCs in the industrial PHA production and lead us to believe that technological advances will allow the achievement of better productivities and lower production costs.

2.7. HSSL

Several advances in genetics, biotechnology and engineering are leading to a new manufacturing concept for converting renewable biomass to valuable fuels and products,

generally referred to as a biorefinery. The American National Renewable Energy Laboratory (NREL) defined a biorefinery as "a facility that integrates biomass conversion processes and equipment to produce fuels, power, and chemicals from biomass. The biorefinery concept is analogous to today's petroleum refineries, which produce multiple fuels and products from petroleum" (NREL 2009). Biorefineries can produce various intermediates and final products, fuel or energy from cheap substrates such as industries wastes from food, agricultural, forestry or paper sectors (Kamm et al 2007). The use of these wastes as substrates can simultaneously allow the reduction of disposal costs of these wastes and the production of high value products (Castilho et al 2009). The lignocellulosic biorefinery uses second-generation technologies to promote the valorisation of wastes from agriculture or forests and presents high potential due to the abundance and accessibility of the lignocellulosic biomass (Galbe and Zacchi 2007; Kamm et al 2007).

Trees can be classified as hard- or softwood. Although the fibers in both types of trees are constituted by the same components (mainly cellulose, hemicellulose and lignin), their proportions and structural characteristics are different (Pereira et al 2013) The substrate used in this work is the HSSL, a by-product of the paper industry. HSSL is a side product from acidic sulphite wood pulping which is usually burned to produce energy. The annual production of bleached sulphite eucalypt pulp is about 1 million tons per year contributing to the economic profits of South Africa, Portugal, and Spain (Marques et al 2009).

In the papermaking process, during grinding of wood, lignin is separated of the remaining fibrous material that is used to produce paper (Pereira et al 2013). This happens during the acid sulphite cooking, where lignin is removed from the remaining wood in the form of lignosulphonates (LS). *Eucalyptus globulus*' HSSL main carbon compounds are LS and phenolic derivatives (60 – 80 g/L), sugars (35 – 45 g/L) from hydrolysed hemicelluloses, mainly xylose, and acetic acid (8 – 9 g/L) (Marques et al 2009). The chemical composition of HSSL is listed in Table 4.

Table 4. Chemical composition of HSSL (Xavier et al 2009)

Components	Concentration (g/L)
LS	78.2 ± 0.6
Acetic acid	8.2 ± 0.3
Furfural	< 0.1
Ash	19.8 ± 0.2
D-Xylose	24.6 ± 0.5
D-Mannose	8.5 ± 0.9
L-Arabinose	7.8 ± 0.3
D-Galactose	4.5 ± 0.1
D-Glucose	2.3 ± 0.1
L-Rhamnose	1.6 ± 0.3
L-Fucose	0.4 ± 0.3

HSSL presents monomeric sugars in high amounts, so it could be considered an ideal candidate for bioprocessing. However, it presents high levels of acetic acid, furfural and low molecular weight LS which inhibit microbial metabolism, affecting the bioprocessing of HSSL (Xavier et al 2009). However, this inhibition is not felt by MMCs.

3. Materials and Methods

3.1. Cultures

3.1.1. SBR

The MMC was collected from an aerobic tank of the WWTP of Aveiro South (SIMRia 2014).

3.1.2. Acidogenic fermentation

The MMC used to inoculate the AFR was collected from the anaerobic digester of the WWTP Aveiro South (SIMRIA 2014). A pre-treatment was performed by heating the inoculum to 82ºC for 20 minutes in order to promote the enrichment of the MMC in acidogenic population over the methanogenic bacteria.

3.2. Bioreactors

3.2.1. AFR

The acidification of HSSL under anaerobic conditions was performed in a CSTR with a working volume 1.55 L and a flow rate of the feeding solution of 0.97 L/d (imposed by an IsmatecTM Compact Digital Multichannel Pump) resulting in a HRT of 1.6 days. The reactor had no system for retaining the biomass; therefore the SRT was the same as the HRT. The effluent was collected at the outlet of the reactor by overflow. Reactor stirring was performed by a magnetic stirrer and kept constant at 100 rpm. Nitrogen was bubbled occasionally to assure anaerobic conditions. Oxidation-reduction potential (ORP) was monitored with a transmitter M300 2-channel, ORP meter (Mettler-Toledo Thornton, Inc). The system worked with temperature control at 30.0 ± 1.0ºC using an external serpentine with meter.

3.2.2. SBR

In this work, a SBR was operated for 70 days in order to select a stable culture capable of storing PHA. The working volume was 1.5 L and the reactor was operated in cycles of ADF. Initially, the cycle duration was 24 hours comprising 22.5 hours of aerobiosis, 1 hour of settling (with agitation and aeration switched off) and 0.5 hours withdrawing of half of the volume of the supernatant (using a Watson-Marlow 101/R

pump), which was replaced by 750 mL of fresh medium during the first 15 minutes of each cycle (with a Watson-Marlow SCI 400 pump). These conditions resulted in a HRT of 2 days. At the end of each cycle, before settling, 150 mL of mixed liquor were removed in order to establish a SRT of 10 days. Thereafter, the cycle's duration was changed to 12 hours, with 10.5 hours of aerobiosis, 1 hour of settling, 0.5 hours of withdrawing and 15 minutes of replacement with fresh medium. With this, the HRT and SRT were kept at 1 and 5 days, respectively. Several timers were used to control the reactor stirring (300 rpm), aeration, feeding and withdrawing. The system was operated without control of pH, temperature and oxygen. Air was supplied through a Boyu Air Pump, 8 L/min. pH values were monitored using a pH meter pH Crison 28-P and the oxygen and temperature values were monitored with Oxygen meter M300 Transmitter meter (Thornton Mettler-Toledo, Inc).

Figure 5. SBR, feeding, aeration pump and feeding and withdrawing pumps; oxygen/temperature and pH meters situated in a laboratory fume hood

3.3. Culture media

3.3.1. Acidogenic fermentation medium

3.3.1.1. HSSL pre-treatment

HSSL from magnesium based acidic sulphite pulping of *Eucalyptus globulus* was supplied by Caima – Indústria de Celulose S.A. (Constância, Portugal). The HSSL

collected from the factory was subjected to a chemical pre-treatment in order to remove some toxic compounds, such as phenolics, furfural and some LS. The pre-treatment of HSSL consisted in pH adjustment to 7.0 with KOH, followed by aeration with compressed air (6 h/L) to oxidize and precipitate some phenolic compounds. Then, the HSSL was centrifuged for 1 hour at 5000 rpm and the precipitated colloids were filtered off using a 1 μm pore size glass microfiber filter. The pre-treated HSSL was stored at 4ºC.

3.3.1.2. Acidogenic fermentation medium

The medium used on the acidogenic CSTR was composed of (per litre of distilled water): 80 mg $CaSO_4 \cdot 2H_2O$, 160 mg $FeSO_4 \cdot 7H_2O$, 80 mg K_2HPO_4, 160 mg KH_2PO_4, 160 mg $MgSO_4 \cdot 7H_2O$, 80 mg $Na_2MoO_4 \cdot 2H_2O$ and 160 mg NH_4Cl. Phosphate salts were prepared apart, in order to avoid the irreversible precipitation with magnesium salts during sterilization. The pH of the feeding, before sterilization, was kept above 6.50. The flasks with medium and phosphates were sterilized in autoclave and phosphates were further added to the alimentation flask in a laminar flow hood. The COD of the feeding was 16 gCOD/L, which corresponded to an OLR of 10.3 gCOD/L.d.

3.3.2. Culture selection and PHA accumulation medium

The medium for culture selection and PHA accumulation steps was prepared using the treated effluent of the acidification reactor supplemented with nutrients. The pre-treatment of acidogenic fermentation reactor effluent consisted in effluent centrifugation for 1 h at 5000 rpm followed by filtration of precipitated colloids using a 1 μm glass microfiber filter (Ahlstrom, grade 131). Finally, the effluent was sterilized in autoclave.

The medium was composed of (per litre of distilled water): 0.080 g $CaSO_4 \cdot 2H_2O$, 0.020 g $FeCl_3$, 0.064 g K_2HPO_4, 0.016 g KH_2PO_4, 0.160 g $MgSO_4 \cdot 7H_2O$, 0.4 g CH_4N_2S, 0.008 g $Na_2MoO_4 \cdot 2H_2O$ and 0.160 g NH_4Cl. Phosphate salts were prepared apart, in order to avoid the irreversible precipitation with magnesium salts during sterilization. To inhibit nitrification, 0.4 g/L of thiourea were added. The flasks with the medium and phosphates were sterilized in autoclave. The pH of the feeding, before sterilization, was kept above 6.50 and the phosphates were further added to the alimentation flask in a laminar flow hood. The COD in the feeding was 2 gCOD/L, which corresponded to an OLR of 1 and 2 gCOD/L.d, in the 24h cycle and 12h cycles, respectively. The average total VFAs content

of the feeding was 1.0 gVFAs/L.

3.4. Sampling

During the motorization of SBR cycles, samples were collected before the feeding period (t = 0), after the feeding stopped (t = 0.25 h) and with 30 minutes periods during the first 2 hours of the cycle. After this, samples were collected every hour during the first 8 hours of the cycle. Samples for determination of total suspended solids (TSS) and volatile suspended solids (VSS) were collected after the feeding and at the 4^{th} and 8^{th} hour of the cycle motorization period. When each sample was collected, pH, temperature and DO percentage of the reactor were registered. After sample collection, pH was measured again (Consort C833). Each sample was centrifuged at 13 000 rpm during 10 minutes (Centrifuge MiniSpin, Eppendorf), and the supernatant was separated from the pellet. The pellet and supernatant were stored in the freezer at -16ºC. The supernatant was used to determine the consumption of acids (acetic, propionic and butyric acids) and sugars (xylose and glucose), chemical oxygen demand (COD), lignosulphonates and ammonia. The lyophilized pellet was used to determine the PHA concentration by Gas Chromatography (GC).

3.5. Accumulation tests

Accumulation tests were performed with several pulses of feeding with the same composition used in the SBR but with some differences. The first test was performed with all the nutrients, the second one without phosphorus and the third one without ammonium.

The kinetic tests with all the nutrients and without ammonia were performed in a bioreactor (BIOSTAT®) operated in fed-batch mode, Figure 6. A respirometer was coupled to the bioreactor by silicon tubing and the recirculation of the medium was performed by a pump (Watson-Marlow SCI 400 pump). The stirring in the reactor was kept constant at 250 rpm, and the supply of air was performed by an air pump (Boyu Air Pump, 8 L/min). The bioreactor was operated without temperature control and sterile conditions. 1 L of inoculum withdrawn from the SBR was used. Five pulses of feeding (each with 500 mL) were supplied, so the final working volume was of 3.5 L. Samples were collected every 15 minutes and the DO percentage was recorded at each 5 seconds for 3 minutes.

Figure 6. Bioreactor used in kinetic test

The kinetic test performed without phosphorus was carried out in a bioreactor with a working volume of 0.5 L without temperature control and sterile conditions, Figure 7. The respirometer was coupled to the bioreactor by silicon tubing and the recirculation of the medium was performed by a peristaltic pump (Gilson Miniplus 3). The stirring in the reactor and respirometer were kept constant, 300 rpm, and the supply of air was performed by an air pump (Boyu Air Pump, 8 L/min). 250 mL of inoculum withdrawn from the SBR were used. Three pulses with 250 mL of feeding without phosphorus were used. Samples were collected every 15 minutes and the DO percentage was recorded at each 5 seconds for 3 minutes.

Figure 7. Reactor, respirometer, oxygen meter and peristaltic pump used in kinetic test

3.6. Analytical Methods

3.6.1. Carbon sources analysis

The consumption of VFAs (acetic, propionic and butyric) and sugars (glucose and xylose) was measured by high performance liquid chromatography (HPLC).

First, samples were filtered using a membrane of 0.22 μm (Corning Costar Spin-X) at 8000 rpm (Centrifuge MiniSpin, Eppendorf) for 15 minutes. pH of samples was corrected to values in the range of 1 – 3, by adding H_2SO_4 0.25 M. HPLC analyses were carried out using an ion exchange column (Aminex HPX-87H) with dimensions of 300×7.5 mm, connected to a pump (HITACHI L-2130 pump), a refractive index detector (Hitachi RI Detector L-2490) and an auto-sampler (Auto-sampler HITACHI L-2200). The column temperature was 40ºC (external oven ParaLab). The eluent was H_2SO_4 0.01 N at a flow rate of 0.6 mL/min and it was prepared with milli-Q water and filtered with a cellulose acetate membrane, 0.2 μm pore size (Whatman).

The injected volume was 20 μL. A calibration using standard curves for glucose, xylose, acetic, propionic and butyric acids was applied, in a concentration range of 0 to 2.0 g/L for glucose, acetic and propionic acids, 0.25 to 5.0 g/L for butyric acid and 0 to 10.0 g/L for xylose.

3.6.2. TSS and VSS determination

Cell dry weight was determined as VSS according to Standard Methods (APHA, 1995). 5 mL of sample was filtered by vacuum filtration using filters (Cellulose Acetate Filter with pores 1 μm, Sartorius) previously weighed. Subsequently, the membranes were placed in an oven at 105°C for 24 hours. After cooling, the membranes were weighed and the biomass was determined in g/L of TSS. Afterwards, the membranes were placed in an oven at 550°C for 2 hours. After cooling the membranes, they were weighed and the concentration of biomass was determined in g/L of VSS.

3.6.3. Ammonia quantification

Ammonia concentrations were determined using a Thermo Scientific Ion Selective Electrode. In order to adjust the ionic strength, to 1 mL of each sample, 100 μL of 1M $MgSO_4$ solution were added. A calibration curve was prepared using ammonia standards with a range of concentrations between 1.00×10^{-1} and 6.25×10^{-4} M. The concentration values of each sample were measured in mV, registered after 5 minutes of measurement.

3.6.4. LS quantification

LS quantification was performed according to Restolho et al. (2009), by measuring the absorbance of the conveniently diluted samples at 273 nm, using a UV spectrophotometer (Shimadzu UVmini-1240) (Restolho et al 2009). The LS concentration was determined using the Beer-Lambert law, with $\varepsilon = 7.41\ g^{-1}cm^{-1}$ (Xavier et al 2009).

3.6.5. COD

COD was measured with the spectrophotometer from the Merck kit according with the Standard Methods APHA 5220 D (APHA, 1995) using a digestion solution with $K_2Cr_2O_7$, $HgSO_4$, H_2SO_4 and H_2O and an acidic solution with H_2SO_4 and $AgSO_4$. Each tube was prepared by adding 1.2 mL of digestion solution and 2.8 mL of acidic solution. Then, 2.0 mL of diluted sample were added. A blank was prepared by adding to the test tube 2.0 mL of distilled water instead of sample. Then, the tubes were shaken and placed in a block digester Spectroquant TR620 (Merck Millipore) pre-heated to 150ºC for 2 h. After cooling down to room temperature, the optical path of the test tubes was carefully cleaned and the absorbance at 600 nm of each sample and blank was measured in a colorimeter

Spectroquant Picco COD/CSB (Merck Millipore). A calibration curve was prepared with standards of glucose and potassium hydrogen phthalate.

3.6.6. PHA

PHA concentrations were determined by GC using the method adapted from Lemos et al. (2006). Lyophilized biomass was incubated for 3.5 h at 100ºC with 1:1 solutions of chloroform with heptadecane as internal standard, and a 20% acidic methanol solution. After the digestion step, the organic phase of each sample was extracted and injected into a gas chromatograph coupled to a Flame Ionization Detector (GC-FID, Konik Instruments HRGC-3000C). A ZBWax-Plus column was used with hydrogen as the carrier gas (50 kPa). Split injection at 280ºC with a split ratio of 1:6 was used. The oven temperature program was as follows: 60ºC; then 20ºC/min until 100ºC; then 3ºC/min until 175ºC; and finally 20ºC/min until 220ºC. The detector temperature was set at 250ºC. HB and HV concentrations were calculated using standards of a commercial P(3HB-*co*-3HV) (88%/12%, Aldrich) and corrected using a heptadecane internal standard.

3.7. Microbial community analysis

3.7.1. Biomass fixation

Samples were centrifuged for 5 minutes at 8000 rpm and the pellet was resuspended in 1×PBS. This step was repeated 3 times. Paraformaldehyde was added to the resuspended biomass, in a proportion of 3 parts of paraformaldehyde to 1 part of biomass. The mixture was incubated for 3 to 12 hours at 4ºC. The fixed sample was centrifuged (5 min, 8000 rpm, 4ºC) and the supernatant was removed. The pellet was resuspended in 1×PBS and this step was repeated 3 times to remove residues of paraformaldehyde. The biomass was resuspended in 1 volume of ice-cold 1×PBS and 1 volume of ice-cold 96% (v/v) ethanol. Samples were stored at – 20ºC (Amann et al 1995).

3.7.2. Gram Staining

After sample fixation, followed by air drying, the microscope slide was stained for 1 minute with crystal violet solution and then the solution was removed with distilled water. After, the slide was treated with lugol solution for 1 minute, washed with distilled water, decolorized with acetone and then dried. Samples were covered with safranin solution for 1 minute and washed with distilled water. The slide was left to air dry and was

then observed under oil immersion at 1000× magnification with direct illumination using a Zeis Axioskop equipped with JVC TK-128OE Color Video Camera (VLC software), Figure 8.

3.7.3. Neisser Staining

Samples were smeared on microscope slides and left to air dry. Then, the slide was covered with a methylene blue and crystal violet solution for 30 seconds and washed with distilled water. After this, the slides were stained with brown bismark solution for 1 minute and then washed with distilled water. The slides were left to air dry and were then observed under oil immersion at 1000× magnification with direct illumination using a Zeiss Axioskop equipped with JVC TK-128OE Color Video Camera, (VLC software), Figure 8.

Figure 8. Optical microscope Zeiss Axioskop equipped with JVC TK-128OE Color Video Camera

3.7.4. Nile Blue Staining

Thin smears of samples were prepared on microscope slides and were left to air dry. Then the slides were dipped in a bath of Nile Blue solution (1% w/v) at 55ºC for 10 minutes. The slides were removed from the bath and washed with 8% acetic acid solution for 1 minute. The slides were left to air dry and were then observed under oil immersion at 1000× magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 9.

3.7.5. Extracellular Polymeric Substances (EPS) Staining

The coloration of α- and β-D-glucopyranose polysaccharides and proteins was prepared separately using different stains specific of each one of these compounds.

3.7.5.1. α-D-glucopyranose Polysaccharides Staining

Sample smears were applied on microscope slides. The sample was stained with Concanavalin A 10 μg/mL solution for 30 to 40 minutes at room temperature. Then the slide was washed with cold Mili-Q water. After air dry, the slides were mounted with VectaShield mounting medium. The slides were then observed under oil immersion at 1000× magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 9. Microscope observation was performed using the CY3 filter (Neu et al 2001).

3.7.5.2.β-D-glucopyranose Polysaccharides Staining

Sample smears were applied on microscope slides. The sample was stained with Calcofluor white (Sigma, St. Louis, USA) 300 mg/L solution for 4 hours at room temperature. Then the slide was washed with cold Mili-Q water. After air dry, the slides were mounted with VectaShield mounting medium. The slides were then observed under oil immersion at 1000× magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 9. Microscope observation was performed using the DAPI filter (de Beer et al 1996).

3.7.5.3. Protein Staining

Sample smears were applied on microscope slides. The sample was stained with fluorescein isothiocyanate (FITC) (Sigma, St. Louis, USA) 10 g/L solution for 3 hours at room temperature. Then the slide was washed with cold Mili-Q water. After air dry, the slide was mounted with Vectashield mounting medium. The slides were then observed under oil immersion at 1000× magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 9. Microscope observation was performed using the FITC filter (Schmid et al 2003).

Figure 9. Epifluorescence microscope Olympus BX51, equipped with an Olympus XM10 camera

3.7.6. FISH

3.7.6.1. Pre-treatments

3.7.6.1.1. Pre-treatment with glass beads

In order to decrease the cellular aggregation of the fixed biomass, small aliquots were transferred to new eppendorfs with glass beads, with a diameter of 1 mm. Eppendorfs were taken to vortex for 60 seconds (Ghosh 2006).

3.7.6.1.2. Pre-treatment with ultrasounds

The fixed biomass was treated with ultrasounds in order to disaggregate the biomass flocs. A small aliquot of sample was transferred to a new eppendorf and then immerged in an ultrasound bath Branson 2510 at 40 kHz for 10 seconds.

3.7.6.2. FISH analysis

5 µL of biomass fixed on paraformaldehyde (4 % v/v) were placed in individual wells of a teflon coated slide and then left to air dry. Next, the slide was dehydrated in ethanol series of 50%, 80% and 98% for 3 min each. Meanwhile, the hybridization and washing buffers were prepared according to stringency of the probe(s) used. 10 µL of hybridization buffer and 0.5 µL of each probe (50 ng/µL) were added to each well. Next, the slide was placed inside the hybridization chamber and the remaining hybridization buffer was used to moisten a tissue paper placed inside this chamber. The hybridization

chamber was incubated at 46ºC for 1.5 hour. The washing buffer was placed in a bath at 48ºC and after the incubation time, the slide was washed with washing buffer, and then placed in a Falcon tube with the remaining washing buffer, for 15 minutes at 48ºC. Then, the slide was washed with cold Milli-Q water and was left to air-dry. After dry, the slides were mounted with Vectashield mounting medium containing DAPI stain (Amann et al 1995). The slides were then observed under oil immersion at 1000× magnification resorting to an epifluorescence microscope, Olympus BX51, equipped with an Olympus XM10 camera (Cell-F software), Figure 9. The list of FISH probes applied is in Table 5.

Table 5. FISH probes used

Probe	Sequence (5' – 3')	Target	References
EUB338	GCTGCCTCCCGTAGGAGT		
EUB338 II	GCAGCCACCCGTAGGTGT	Bacteria	(Amann et al 1995)
EUB338 III	GCTGCCACCCGTAGGTGT		
Delta495a	AGTTAGCCGGTGCTTCTT		
Delta495b	AGTTAGCCGGCGCTTCCT	*Deltaproteobacteria*	(Loy et al 2002; Lücker et al 2007)
Delta495c	AATTAGCCGGTGCTTCCT		
Lgc354a	TGGAAGATTCCCTACTGC	*Firmicutes* (Gram$^+$ bacteria with low GC content)	
Lgc354b	CGGAAGATTCCCTACTGC		(Meier et al 1999)
Lgc354c	CCGAAGATTCCCTACTGC		
Gnsb941	AAACCACACGCTCCGCT	*Chloroflexi* (green nonsulfur bacteria)	(Gich et al 2001)
Alf968	GGTAAGGTTCTGCGCGTT	*Alphaproteobacteria* (except Rickettsiales)	(Neef 1997)
Bet42a	GCCTTCCCACTTCGTTT	*Betaproteobacteria*	(Manz et al 1992)
Gam2a	GCCTTCCCACATCGTTT	*Gammaproteobacteria*	(Manz et al 1992)
Hgc69a	TATAGTTACCACCGCCGT	*Actinobacteria* (high GC Gram$^+$ bacteria)	(Roller et al 1994)
Pla46	GACTTGCATGCCTAATCC	*Planctomycetales*	(Neef et al 1998)
Cf319a	TGGTCCGTGTCTCAGTAC	*Flavobacteria*, *Bacteroidetes*, *Sphingobacteria*	(Manz et al 1996)
Arc915	GTGCTCCCCCGCCAATTCCT	*Archaea*	(Stahl and Amann 1991)
TM7905	CCGTCAATTCCTTTATGTTTTA	Candidate division TM7	(Hugenholtz et al 2001)
DF988*	GATACGACGCCCATGTCAAGGG	*Defluvicoccus*	(Meyer et al 2006)
DF1020*	CCGGCCGAACCGACTCCC		
TFO-DF218	GAAGCCTTTGCCCCTCAG	*Defluvicoccus* related TFO	(Wong et al 2004)
TFO-DF618	GCCTCACTTGTCTAACCG		
SBR9-1a	AAGCGCAAGTTCCCAGGTTG	*Sphingomonas*	(Beer et al 2004)
THAU646	TCTGCCGTACTCTAGCCTT	*Thauera sp.*	(Lajoie et al 2000)
AZO644	GCCGTACTCTAGCCGTGC	*Azoarcus sp.*	(Hess et al 1997)
PAR651	ACCTCTCTCGAACTCCAG	*Paracoccus*	(Neef et al 1996)
AMAR839	CCGAACGGCAAGCCACAGCGTC	*Amaricoccus sp.*	(Maszenan et al 2000)
ACI145	TTTCGCTTCGTTATCCCC	*Acidovorax* spp.	(Schulze et al 1999)

3.7.7. Extraction, Purification and Amplification of genomic DNA

DNA was extracted from a SBR sample collected during the apparent stationary phase of operation (on 66th day). DNA extraction was performed using PowerSoil® DNA Isolation kit following the protocol described by the manufacturer.

The primers used to amplify 16S rDNA were 27 forward (f) and 1492 reverse (r), Table 6. Polymerase Chain Reaction (PCR) was performed in the BioRad T100™ Thermal Cycler. For the 27f and 1492r primers, a reaction volume of 25 μL was used and the PCR reaction was composed by: 12.5 μL of Lucigen Taq98™ Hot Start 2X master mix, 1.25 μL of each primer, 1 μL of DNA and 9 μL of nuclease-free water. The following PCR cycles were used: 98ºC for 2 minutes, followed by 35 cycles of 98ºC for 0.5 minutes, 58ºC for 0.5 minutes and 72ºC for 1 minute and, finally, an extension at 72ºC for 10 minutes. After the last cycle, samples were cooled down to 4ºC and an agarose (1%) electrophoresis was performed to check if there was amplification. Afterwards, the PCR products were purified using the QIAquick® PCR purification kit of (Quiagen, Milan, Italy) and quantified using the NanoDrop2000 Spectrophotometer (ThermoScientific, Milan, Italy).

3.7.8. 16S rDNA Clone Library

After purification and quantification, the amplified 16S rDNA was ligated into pGEM®-T vector (Promega, USA) and transformed into JM109 Competent Cells (Promega, USA), according to the manufacturer's instructions. The transformed cells were plated in LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37ºC. Clones were screened for insertion of the correct size by PCR amplification using primers T7f and M13r. A denaturation step was performed before PCR amplification. This step consisted in a cycle of 96ºC for 10 minutes. For the T7f and M13r primers, a reaction volume of 100 μL was used and the PCR reaction was composed by: 50 μL of Lucigen Taq98™ Hot Start 2X master mix, 5 μL of each primer, 40 μL of nuclease-free water and DNA from colony. PCR amplification was performed as already described, using the following cycles: 98ºC for 2 minutes, followed by 35 cycles of 98ºC for 0.5 minutes, 57ºC for 1 minute and 72ºC for 2 minutes and an extension of step of 72ºC for 20 minutes.

DNA sequencing was performed by BioFab (Rome, Italy) using the primers 530f, 926f and 907r, Table 6. In a first approach, all clones were partially sequenced, using 530f primer. After this, taking into consideration the obtained results, some clones were

completely sequenced, using 926f and 907r primers. After receiving the results, the sequences were manually assembled and the organisms' identities were deduced through basic local alignment search tool (BLAST). The complete sequences obtained were deposited in GenBank (accession numbers: KT262954, KT262955, KT262951, KT262956, KT262952, KT262957, KT262958, KT262959, KT262960, KT262961, KT262962, KT262953, KT262963, KT262964, KT2629).

Table 6. Primers used for PCR amplification and sequencing

	Primers	**Sequence (5' – 3')**
PCR	27f	AGAGTTTGATCMTGGCTCAG
	1492r	TACGGYTACCTTGTTACGACTT
	T7f	TAATACGACTCACTATAGGG
	M13r	TCACACAGGAAACAGCTATGAC
Sequencing	530f	GTGCCAGCMGCCGCGG
	926f	AAACTYAAAKGAATTGACGG
	907r	CCGTCAATTCMTTTRAGTTT

4. Calculation of Kinetic and Stoichiometric Parameters

The sludge PHA content was calculated as a percentage of TSS on a mass basis:

$$\%\text{PHA} = \frac{\text{PHA}}{\text{TSS}} \times 100 \ \ (\text{gPHA/gTSS})$$

where TSS includes active biomass (X) and PHA. Active biomass was calculated by subtracting PHA from VSS. The maximum specific substrate uptake ($-q_S$ in CmmolS/CmmolX.h) was determined by adjusting a linear function to the experimental data of substrate concentrations plotted over time, calculating the first derivative at time zero (taking the slope of the fitting) and dividing the value obtained by the active biomass concentration at that point. The volumetric substrate uptakes ($-r_S$ in CmmolS/L.h) were determined by adjusting a linear function to the experimental data of substrate (VFAs) plotted over time, calculating the first derivative at time zero (taking the slope of the fitting). The amount of stored PHA (Δ%PHA) was calculated as the difference between the PHA percentage at the end of the feast phase (i.e. the substrate depletion time) and at the beginning of the respective cycle. The yields of PHA ($Y_{PHA/S}$ in CmmolPHA/CmmolS) and active biomass ($Y_{X/S}$ in CmmolX/CmmolS) on substrate consumed were calculated by dividing the amount of PHA formed or the active biomass formed by the total amount of substrate consumed, respectively. The respiration yield on substrate ($Y_{O2/S}$ in gO_2/gS) was calculated by dividing the decreased amount of O_2 consumed by respiration (in g/L) per amount of substrate consumed (in gS/L). Since no nitrification occurred, because thiurea was added to the feeding medium, and based on the premise that the molecular formula for biomass is $C_5H_7NO_2$, it was possible to establish that for the production 8 mg of active biomass there is a need of 1 mg of N (Serafim et al 2004). This relation allowed for the calculation of the specific growth rate of the active biomass. The specific growth rate was calculated resorting to the following formula:

$$\mu = \frac{1}{\text{X}} \times \frac{dX}{dt}$$

5. Results and Discussion

5.1. SBR performance

The SBR was operated along 70 days with the objective of selecting a stable PHA-producing MMC. During the operational time of the SBR several parameters were monitored such as: consumption of VFAs (acetic, propionic and butyric acids), sugars (xylose and glucose), COD, LS and ammonium and production of PHAs and biomass. The SBR was firstly operated with 24 h cycles and on day 44 the cycle duration was changed to 12 h, in order to increase the number of cycles per SRT.

In this work, the process of culture selection was performed using a medium enriched with VFAs as substrate, since VFAs are direct substrates for PHA storage. The evolution of the uptake rates of acetic, propionic and butyric acids is shown in Figure 10. The average acetic acid uptake rate (2.99 ± 2.02 Cmmol/L.h) was higher that the average uptake rates of propionic acid (1.23 ± 1.13 Cmmol/L.h) and butyric acid (2.19 ± 1.65 Cmmol/L.h), which demonstrates a preference of the culture for acetic acid. The propionic acid consumption was relatively constant throughout the SBR operational time and the butyric acid uptake rate stabilized from day 37. Ammonium was consumed at 0.111 ± 0.100 g/L.h. Between the 32nd and 52nd day, the ammonium uptake rates suffered an increase, and the highest value was recorded on the 52nd day (0.339 g/L.h).

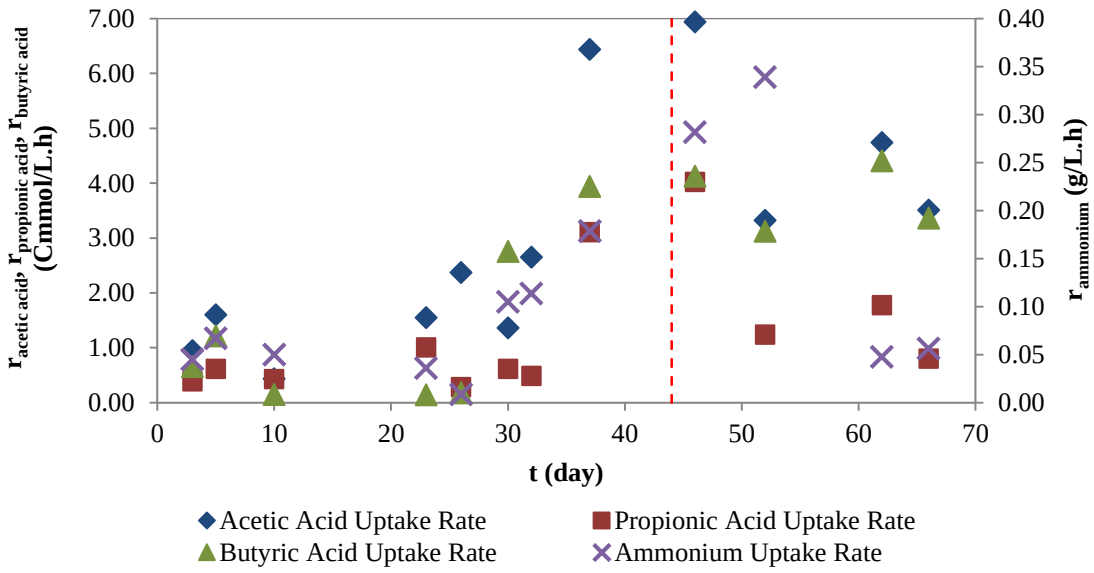


Figure 10. Acetic, Propionic and Butyric Acids and Ammonium uptake rates during the SBR operation. Day 0 to 42: 24 h cycle; Day 42 to 66: 12 h cycle

Figure 11 shows the evolution of Δ%PHA, active biomass and PHA concentrations along the SBR operational time. The average variation of Δ%PHA was 16.2 ± 13.9%, with the lowest value being registered at the 34th day (0.34%) and the highest value at the 10th day (48.8%). The values of Δ%PHA and PHA concentration were unstable throughout the first 37th days of SBR operation and several peaks of production were detected. Between the 46th and 66th day, although Δ%PHA was lower than before, PHA production became more stable and it varied in the range 9.1 ± 2.4%. This leads to believe that the culture PHA storage was not affected by change of cycle duration.

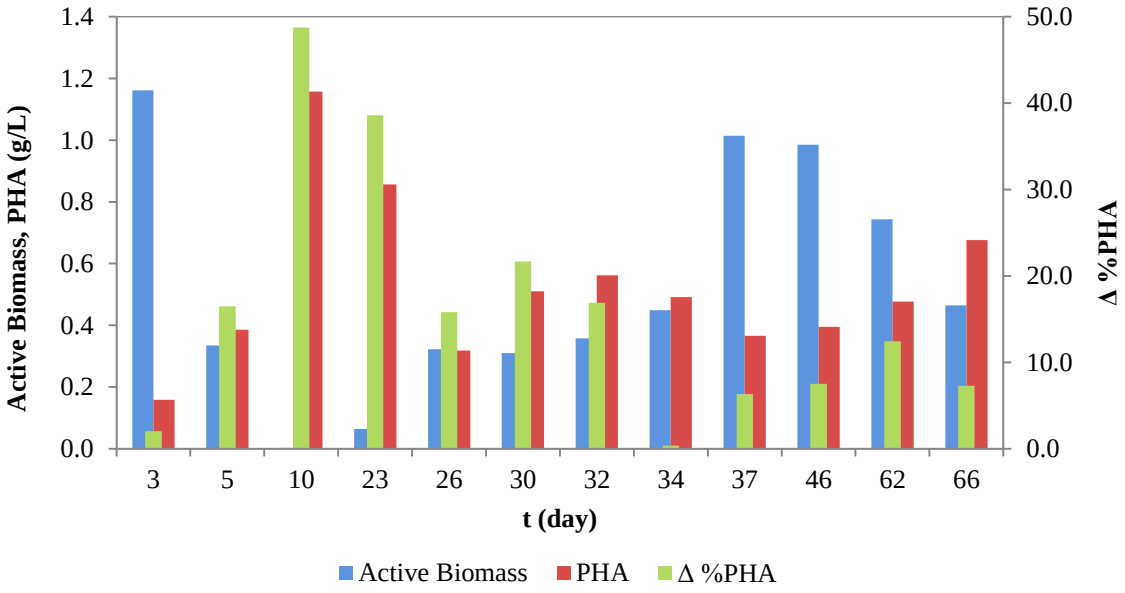


Figure 11. Δ%PHA, Active Biomass and PHA concentrations throughout the SBR operational time

Figure 12 shows the evolution of the produced monomers during the SBR operation. If mixtures of VFAs such as acetate, propionate, butyrate, and valerate are used as a substrate for PHA production, acetyl-CoA, and propionyl-CoA are formed as precursors for PHA production, resulting in a copolymer containing HB and HV monomers (Dias et al 2006; Albuquerque et al 2007). In this case, the produced PHA was a copolymer P(3HB-*co*-3HV) with an average monomeric composition (on a molar basis) of 69:31 ± 7:7 (HB:HV). After the change of cycle duration on day 44, the co-polymer composition became more stable, having a average monomeric composition (on a molar basis) of 77:23 ± 1:1 (HB:HV).

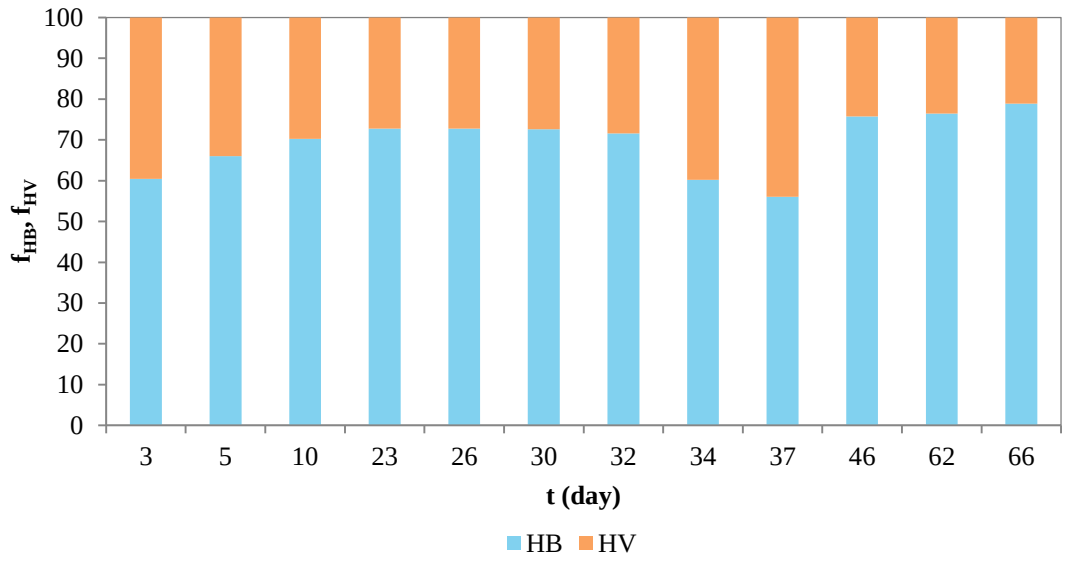


Figure 12. Evolution of the content of HB and HV during the SBR operational time

The F/F ratio is an important parameter in the culture selection. In general, low F/F values ensure physiological adaptation of the microorganisms, favouring PHA storage during the feast phase (Albuquerque et al 2011; Reis et al 2011). As shown in Figure 13, around day 34, the F/F ratio stabilized at 0.091, which is an indication of culture stabilization. On day 44, the cycle duration was changed and after some instability, the F/F ratio stabilized again on the 55th day, this time at 0.2. This corresponded with the stabilization of VFAs uptake rates and with the stabilization of PHA production. According to Dionisi et al. (2007), the best storage response is observed when the feast phase is not longer than 20% of the length of the cycle, so the obtained F/F ratio is considered acceptable. Albuquerque et al. (2010b) also found a predominant storage response with a F/F ratio of 0.21, using fermented molasses as substrate.

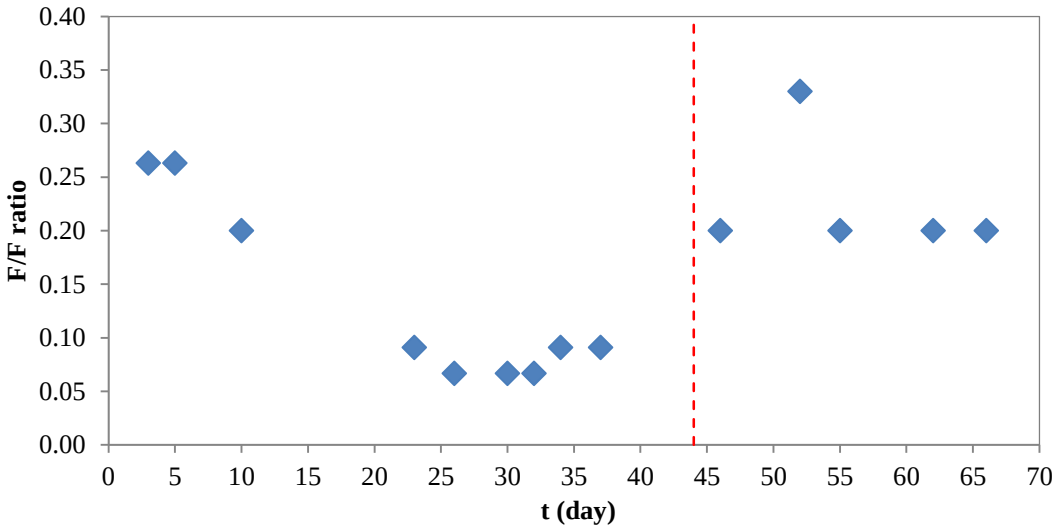


Figure 13. F/F ratio throughout the SBR operational period.
Day 0 to 42: 24 h cycle; Day 42 to 66: 12 h cycle

5.1.1. SBR cycles

Along the 70 days of SBR operation, several cycles were monitored in order to follow the evolution of the culture selection process. During cycles several parameters were followed, such as the pH, temperature, uptake of acetate, propionate, butyrate and ammonium, oxygen consumption and PHA production.

5.1.1.1. 24 hour cycle period

Figure 14 shows a representative cycle on day 37, with a F/F of 0.09 and 24 h of cycle length. This cycle was monitored during its first 8 hours, in order to follow the entire feast phase and the beginning of the famine phase.

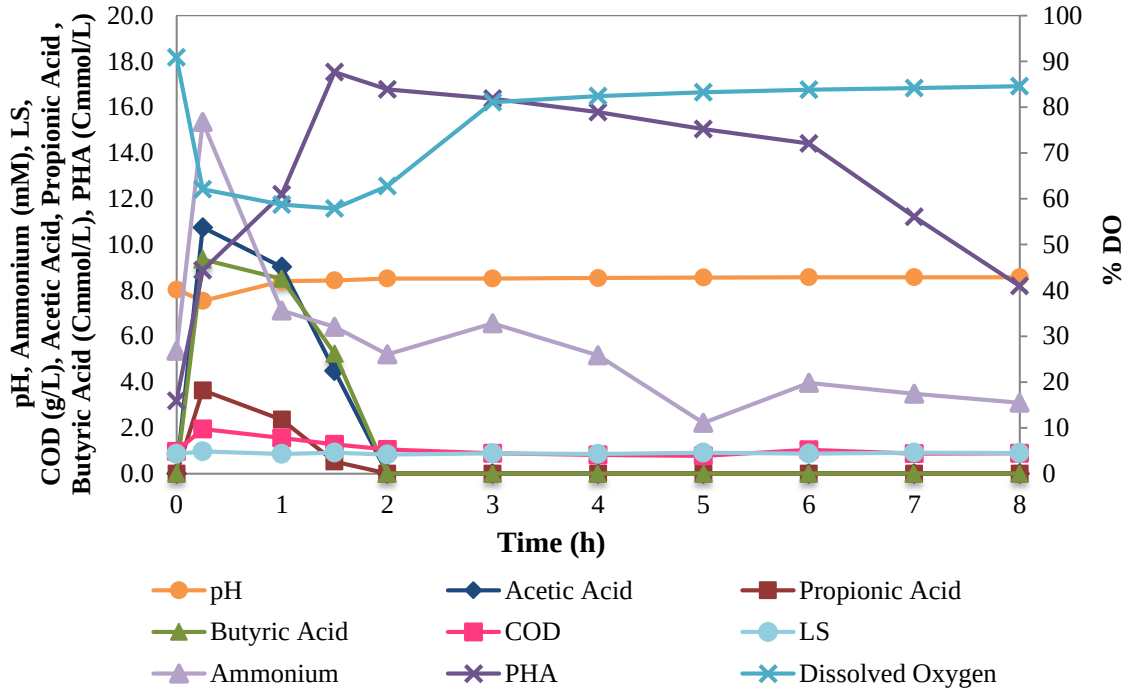


Figure 14. SBR cycle on the 37th day of operation

In the beginning of the cycle, fresh medium was supplied to the reactor (0 – 0.25 h), marking the start of the feast phase. In this work, no pH control was imposed and because of that the pH suffered changes throughout the cycle. It is possible to see that the pH decreased to 7.54 after the feeding (t=0.25 h) and after the first 2 hours, it increased until reaching 8.58. pH increased during the feast phase and then stabilized. Chua and co-workers (2003) studied the effect of pH on PHA content using acetate as substrate. They found that, when controlling the pH at 6 or 7, the PHA content was lower than at pH 8 or 9 (Chua et al 2003). Serafim et al. (2004) also found that the polymer yield per substrate and the intracellular P(3HB) content were higher at pH 8 than at pH 7, and increased sharply when the pH was not controlled (it varied from 8 to 9.5). Oehmen and co-workers (2014) also studied the effect of pH control in the volumetric productivity of PHA production by an MMC (Oehmen et al 2014). The operation of a SBR with pH control at 8 resulted in higher volumetric productivity of PHA as compared to SBR operation without pH control (pH 8–9). The culture selected at pH 8 demonstrated a higher total volumetric productivity of PHA, since it was able to achieve a similar specific PHA production capacity while exhibiting a faster biomass growth rate. Change in pH was found to directly affect the microbial community composition and culture metabolism and *Azoarcus* was more

selectively enriched at pH 8. While controlling pH may present an attractive strategy to increase volumetric PHA productivity, it should be noted that this also implies an additional cost associated with the use of chemicals for pH control in large-scale applications. Nevertheless, from the point of view of the operation simplicity, reactor operation without pH control reduces both process control complexity and operating costs.

The concentrations of acetate, propionate, butyrate, ammonium and LS increased after the feeding, as expected. It is possible to see that the depletion of acetic, propionic and butyric acids occurred after 2 hours. Acetic acid was consumed preferentially with an uptake rate of 6.44 Cmmol/L.h, followed by butyric and propionic acids, with uptake rates of 3.94 and 3.11 Cmmol/L.h, respectively. VFAs were used by bacteria for the formation of new biomass (indicated by ammonia and biomass measurements), but mostly for storage of substrate in form of PHA. During the first 2 hours of the cycle, COD and LS were consumed at 0.584 and 0.023 g/L.h, respectively. During the feast phase, ammonium was consumed at rate of 0.179 g/L.h.

In general, the moment of carbon source exhaustion (end of feast phase and beginning the famine phase) is accompanied by a sudden increase of DO concentration and by oxygen uptake rate (OUR) decrease (Serafim et al 2004). In this case an increase in DO values was verified at the 2nd hour, which coincided with the depletion of acetic, propionic and butyric acids. For this reason, when the acids are exhausted, cells began to consume other available carbon sources that are more difficult to assimilate. If there are no other available substrates, when the famine phase begins, only cells that have accumulated PHA in the feast phase will survive, selecting the best PHA-accumulating microorganisms.

After depletion of VFAs, the concentration of CQO and LS continued to decrease, which indicates that other forms of carbon continued to be consumed by the culture. However, the feast and famine phases are only applicable to organisms that consume VFAs, and are not true feast and famine phases for organisms that consume other carbon sources present, like xylose, glucose and LS. In this cycle, it is possible to define that the feast phase occurred between the 0.25 and 2 hours and that the famine phase occurred between the 3rd and 24th hours. Therefore, this cycle had a F/F ratio of 0.09.

Since VFAs are the preferential substrates for PHA accumulation, it makes sense that the maximum PHA value (17.5 Cmmol/L) was registered at the end of the feast phase, when the depletion of VFAs occurred. For this cycle, the Δ%PHA was 6.3%. After 1.5

hours, the concentration of PHA began to decrease since during the famine phase the culture consumed stored PHA as carbon source to ensure cell growth and maintenance. Although ammonium was consumed at a lower rate during the famine phase (0.041 g/L.h), the presence of ammonium throughout the full feast and famine cycle allowed PHA-accumulating organisms to use the intracellularly stored polymer for growth. The yield $Y_{PHA/S}$ was 0.61 Cmmol PHA/Cmmol S, where S is the sum of VFAs concentrations. Between the 1.5th and 8th hour of the cycle, PHA concentration decreased 53%.

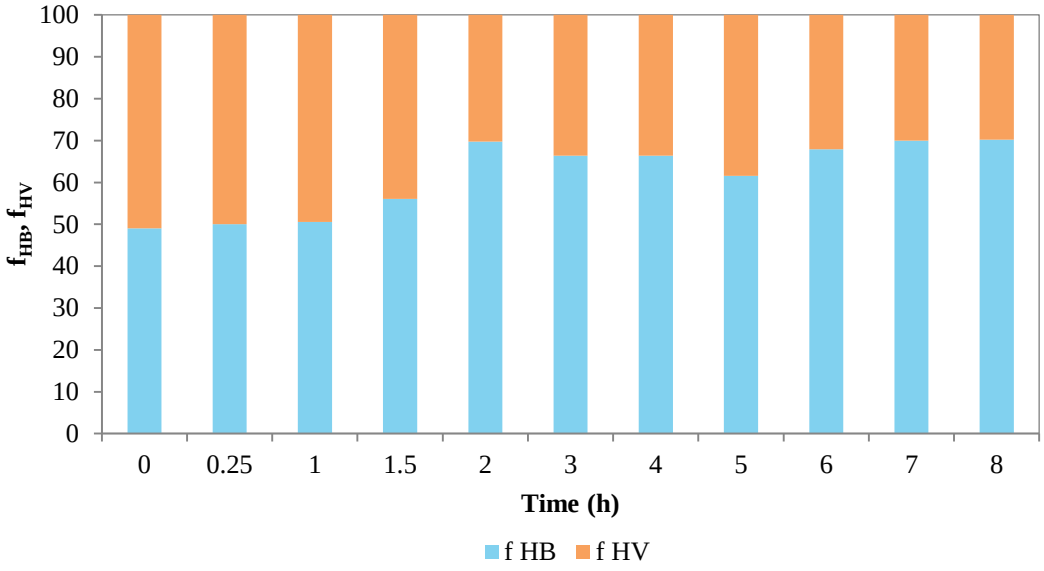


Figure 15. HB and HV monomers molar fraction evolution throughout the cycle from day 37

Figure 15 shows the evolution of the produced monomers during the cycle from day 37. In this case, the produced PHA was P(3HB-*co*-3HV). During the first 2 hours of the cycle, the HB molar fraction suffered an increased and from then on the copolymer composition became stable. At the 1.5th hour, the copolymer had a monomeric composition (on a molar basis) of 56:44 (HB:HV).

5.1.1.2. 12 hour cycle period

Several cycle lengths between 2 and 12 h were already tested for the selection of PHA-producing MMC (Reis et al 2011). In this work, the SBR was firstly operated in 24 h cycles and when a stabilization of the F/F values was observed, the cycle duration was changed to 12 h, in order to increase the number of cycles per SRT. Figure 16 shows the evolution of the various parameters monitored in the SBR cycle.

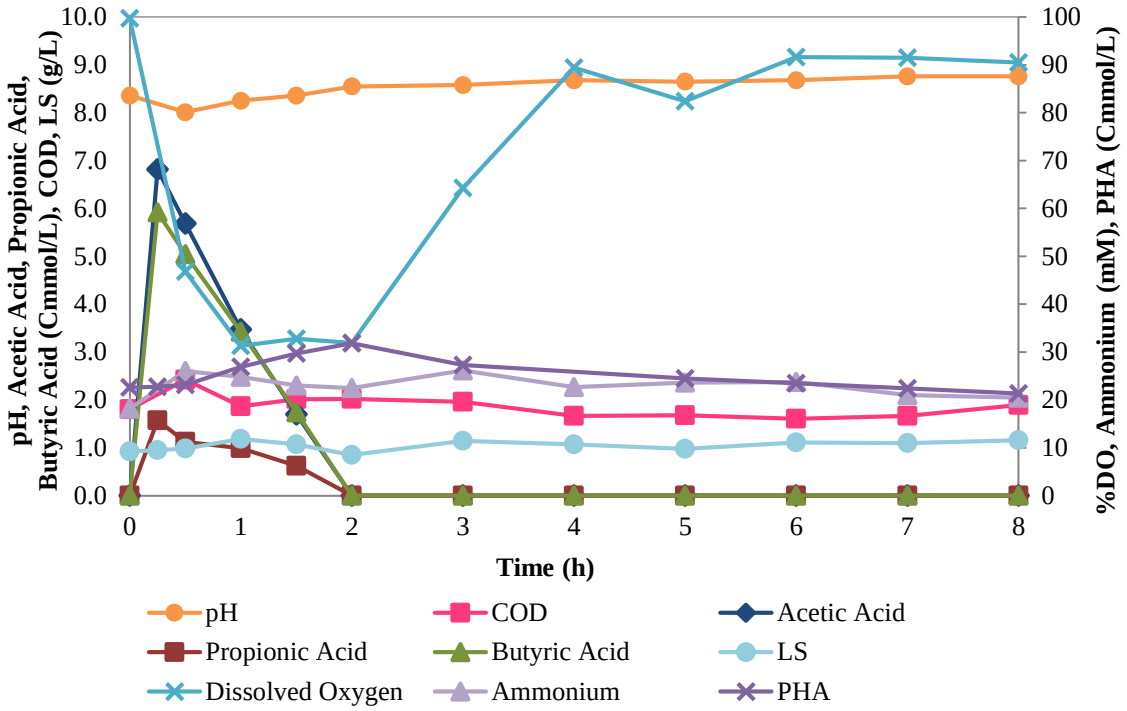


Figure 16. SBR cycle from the 66th day of operation

Regarding pH, the same tendency of the 24 h cycle was observed. The pH decreased to 8.01 after the feeding (t=0.25 h) and then increased until reaching 8.78.

The concentrations of acetate, propionate, butyrate, ammonium and LS increased after the feeding and then declined throughout the cycle. Acetic and butyric acids were consumed preferentially with uptake rates of 3.51 and 3.36 Cmmol/L.h, respectively. Propionic acid was consumed at 0.802 Cmmol/L.h. The same tendencies of VFAs consumption were observed in the 24 h cycle. During the first 2 hours of the cycle, COD was consumed at 0.276 g/L.h. No tendency on LS consumption was observed, so a consumption rate cannot be estimated. During the feast phase, ammonium was consumed at rate of 0.057 g/L.h.

When the substrate was depleted, the famine phase started at the 2nd hour. In this cycle the feast phase occurred between the 0.25 and 2 hours and the famine phase occurred between the 3rd and 12th hours, originating a F/F ratio of 0.2.

The maximum PHA concentration (31.8 Cmmol/L) was registered at the 2nd hour, which corresponded to the end of the feast phase. For this cycle, Δ%PHA was 7.3%. After the 2nd hour, the concentration of PHA began to decrease since the stored PHA was consumed for growth and maintenance. The yield $Y_{PHA/S}$ was 0.64 Cmmol PHA/Cmmol S.

The PHA concentration decreased 33% between the 2nd and 8th hour of the cycle.

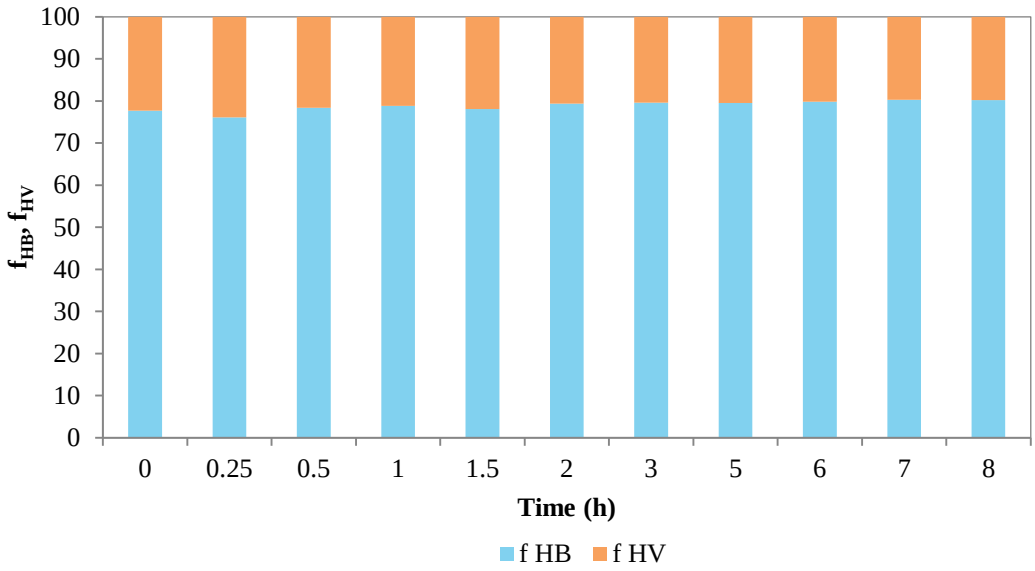


Figure 17. HB and HV monomers molar fraction evolution throughout the cycle from day 66

Figure 17 shows the evolution of the produced monomers during the cycle from the 66th day. At the 2nd hour, the produced copolymer had a monomeric composition (on a molar basis) of 79:21 (HB:HV). The composition of the copolymer was uniform throughout the cycle. When comparing these results with the ones from 24 h cycle, is possible to see that polymer composition was more stable in 12 h cycle. In this cycle, an increase in the HB content was observed which was not expected since the butyric acid proportions used in the feeding, were the same in both cycles.

5.2. Accumulation tests

After operating the SBR under feast and famine conditions for the enrichment in PHA-producing organisms, the storage response of the selected consortia was exploited in the third stage, which consisted in several accumulation tests operated in batch conditions. Three SBR purges from the previous days were used as inoculum for the accumulation tests. Since the objective of these tests was to evaluate the PHA production of the selected culture, the MMC was submitted to consecutive feeding pulses of VFA-rich medium, with the objective of maximizing PHA production. The effects of ammonium and phosphorus limitation were also studied.

5.2.1. Accumulation test without limitations

The first kinetic test was performed with five pulses of feeding with the same composition used in the SBR without nutrient limitation, Figure 18. The kinetic and stoichiometric parameters are shown in Table 7. Five pulses of feeding were supplied to the bioreactor. Each pulse was added when increases in DO was detected, since it is associated with VFAs depletion. The addiction of a new pulse was associated with increases in VFAs, COD and ammonium concentrations and decreases of pH and DO concentration.

Propionic and butyric acids were consumed to exhaustion in every pulse, but the contrary occurred with acetic acid. Butyric acid was preferably consumed, followed by acetic and propionic acids. In general, the moment of carbon source exhaustion is accompanied by a sudden increase of the DO concentration and by the OUR decrease (Serafim et al 2004). However, at the end of each of the five pulses, acetic acid was never depleted. The end of the pulses was only associated with a clear increase in the DO and exhaustion of butyric and propionic acids, which were present in the feeding in lower concentrations.

The maximum PHA content obtained in this assay was 47.1% of cell dry weight at 3.75 h, which is more 68.7% than at the beginning of the experiment. The overall yield $Y_{PHA/S}$ obtained in this accumulation test was 0.39 CmmolPHA/CmmolS. Although the acetic acid was not depleted in any of the pulses, the yield $Y_{PHA/S}$ from the 2^{nd} and 3^{rd} pulses was higher than one (1.63 and 1.05 CmmolPHA/CmmolS, respectively). This is due to the fact that other non-quantified components of the medium, such as LS and phenolics, were probably converted to PHA. The produced copolymer had an average monomeric composition (on a molar basis) of 82:18 ± 2:2 (HB:HV). The monomeric composition of the copolymer was uniform throughout the test.

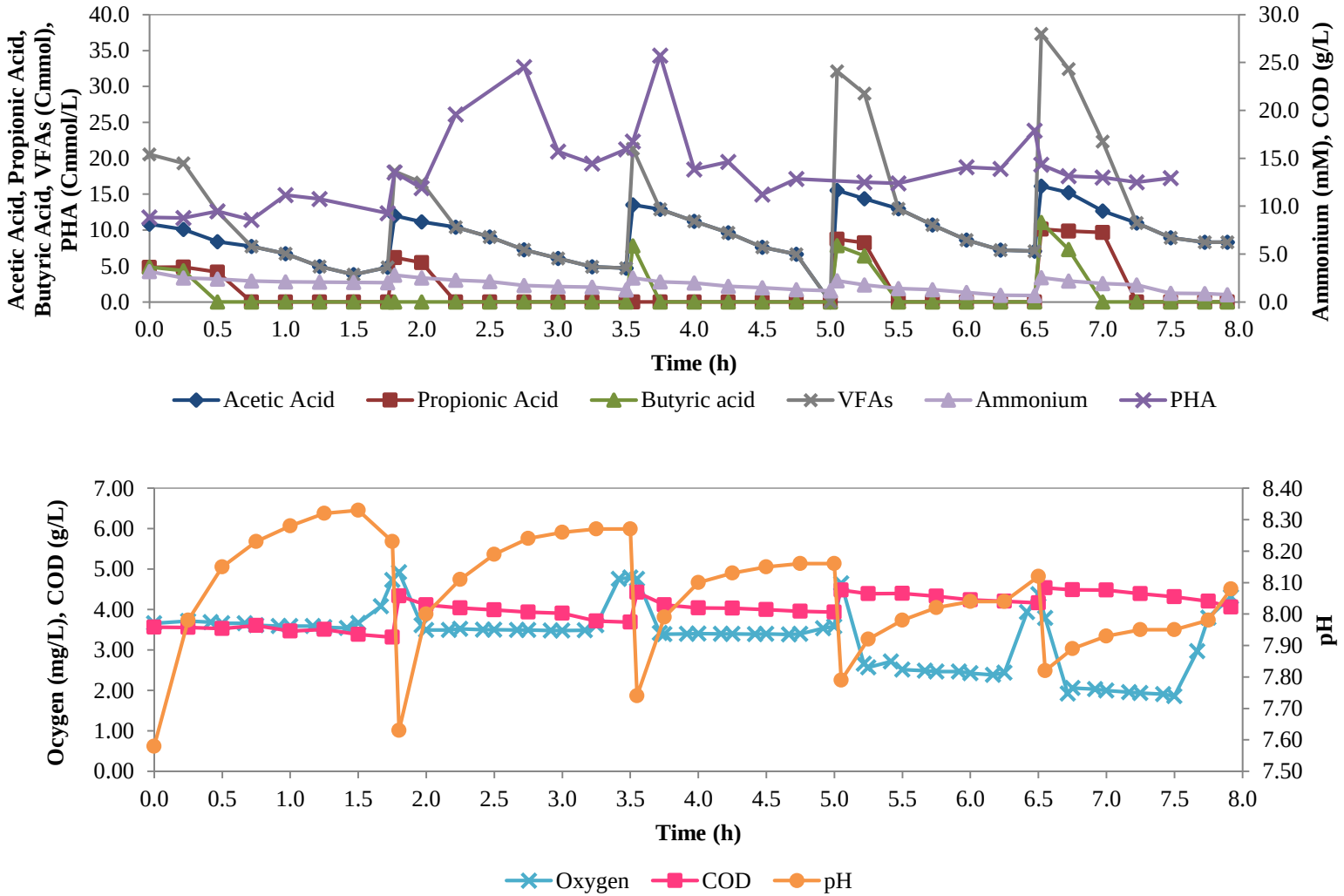


Figure 18. Evolution of pH, Oxygen, Ammonium, COD, PHA Acetic Acid, Propionic Acid, Butyric Acid and VFAs concentrations in kinetic test performed without limitations. Test performed on the 54th day of operation

Table 7. Kinetic parameters obtained in the different accumulation tests

Test	Uptake Rates (Cmmol/L.h)			μ	$Y_{PHA/S}$	$Y_{X/S}$
	Acetic acid	Propionic Acid	Butyric Acid	(h^{-1})	(CmmolPHA/CmmolS)	(CmmolX/CmmolS)
Without limitations	2.82[a]	0.895[a]	1.35[a]	0.103[a]	0.209[a]	0.288[a]
	2.05[b]	1.745[b]	-	0.102[b]	1.63[b]	0.322[b]
	2.28[c]	-	4.95[c]	0.112[c]	1.05[c]	0.304[c]
	2.43[d]	0.786[d]	5.06[d]	0.121[d]	0.607[d]	0.186[d]
	2.23[e]	0.288[e]	5.36[e]	0.120[e]	-	0.411[e]
- Phosphorus	2.47[a]	0.91[a]	1.42[a]	0.108[a]	0.206[a]	0.255[a]
	2.69[b]	0.82[b]	1.89[b]	0.087[b]	0.817[b]	0.258[b]
	1.18[c]	0.99[c]	1.28[c]	0.084[c]	0.964[c]	0.116[c]
- Ammonium	3.92[a]	1.24[a]	1.53[a]	0.078[a]	0.804[a]	0.131[a]
	3.14[b]	1.75[b]	2.60[b]	0.035[b]	1.39[b]	0.148[b]
	3.38[c]	1.48[c]	2.14[c]	0.051[c]	0.955[c]	0.295[c]
	2.87[d]	1.03[d]	6.26[d]	0.021[d]	1.69[d]	0.211[d]
	4.89[e]	1.27[e]	3.05[e]	0.012[e]	-	0.253[e]

a – first pulse, b - second pulse, c - third pulse, d - fourth pulse, e - fifth pulse

5.2.2. Accumulation test with phosphorus limitation

Under phosphorus limitation, phosphorus is supposed to be more transferable than ammonium from one component of the cell to another, due to its reorganization in the cells. Moreover, under phosphorus limitation, ATP synthase activity decreases and the Krebs cycle is restrained, promoting the conversion of excess carbon into PHA. PHA production by axenic cultures appears to be higher under phosphorus limitation than under ammonium limitation (Cavaillé et al 2013). For this motives, phosphorus limitation was tested in an accumulation test. The results obtained are shown in Figure 19 and kinetic and stoichiometric parameters in Table 7. Three pulses of feeding without phosphorus were supplied to the bioreactor. As in the previous assay, only the butyric and propionic acids were consumed to exhaustion in all the pulses, while acetic acid was never exhausted. However, the increase in the DO that generally follows substrate depletion was detected at the end of each pulse. Nevertheless, acetic acid was preferably consumed, followed by butyric and propionic acids.

Growth rates in this kinetic test were lower than those obtained in the accumulation test without limitations. This was expected since, under phosphorus limitation, less carbon is deviated to growth being used for PHA storage.

The maximum PHA content obtained was 32.7% of cell dry weight at 4 h, which was more 57.6% than at the beginning of the experiment. Although acetic acid was always available, during all the pulses the same tendency was observed: first an increase of PHA content occurred, followed by its decrease. The overall yield $Y_{PHA/S}$ obtained in this accumulation test was 0.34 CmmolPHA/CmmolS. The produced copolymer had an average monomeric composition (on a molar basis) of 71:29 ± 12:12 (HB:HV).

Cavaillé et al. (2013) investigated P(3HB) production by waste activated sludge in aerobic fed-batch conditions using acetic acid as substrate, under phosphorus limitation. A maximum P(3HB) content of 67% ($g_{P(3HB)}/gVSS$) and P(3HB) yields ranging from 0.21 to 0.31 Cmmol P(3HB)/Cmmol S were obtained. Although, the maximum PHA content obtained in this accumulation test was lower than the one obtained by Cavaillé and co-workers, the yield $Y_{PHA/S}$ was higher.

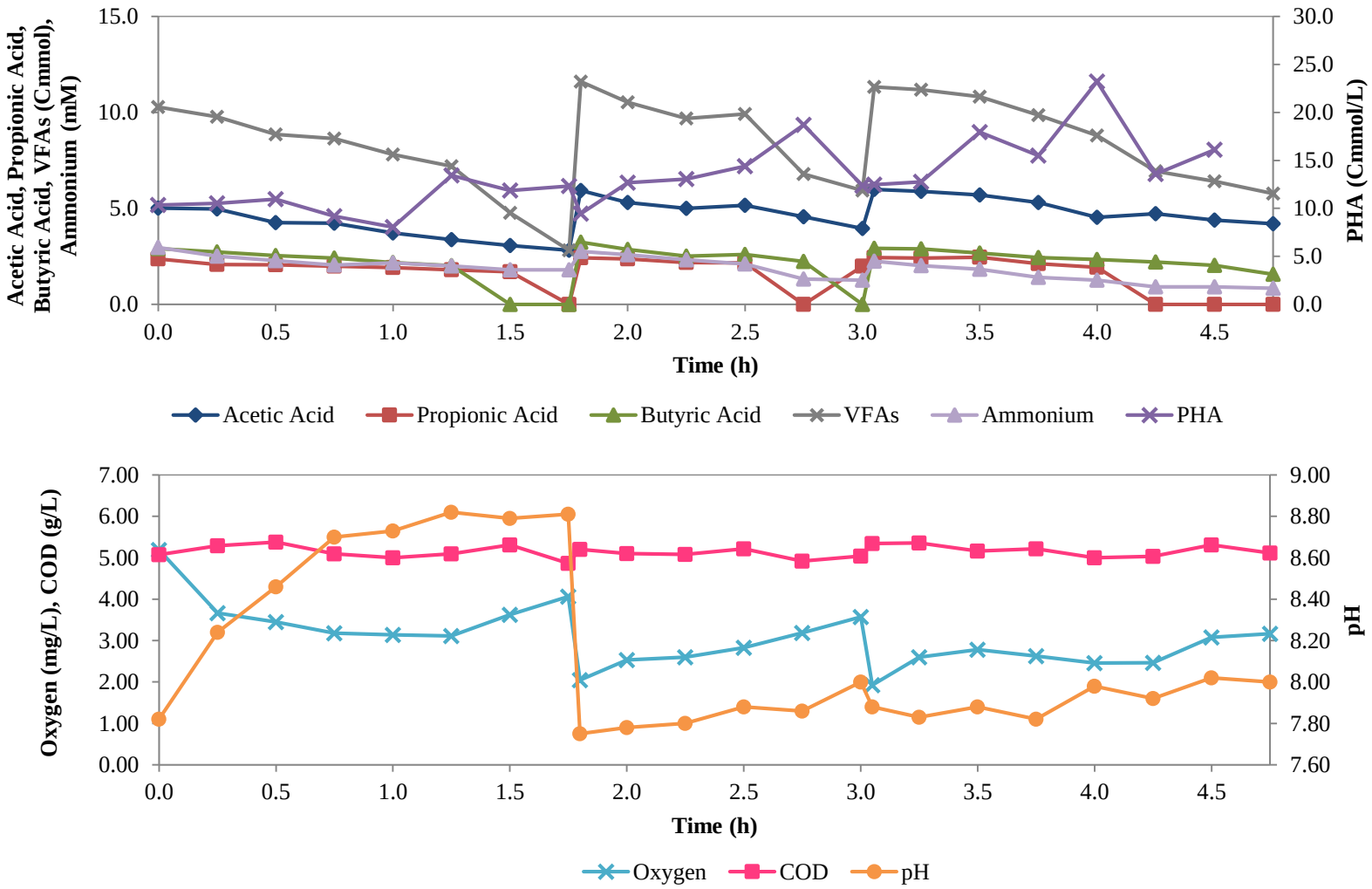


Figure 19. Evolution of pH, Oxygen, Ammonium, COD, PHA, Acetic Acid, Propionic Acid, Butyric Acid and VFAs concentrations in kinetic test performed with phosphorus limitation. Test performed on the 61st day of operation

5.2.3. Accumulation test with ammonium limitation

Ammonium limitation usually triggers PHA production by MMC mixed cultures. Under ammonium limitation, protein synthesis decreases and the carbon flow is redirected to PHA synthesis. Ammonium limitation impacts the cell growth by reducing enzymatic activity (Cavaillé et al 2013). Based on these findings, the production of PHA under ammonium limitation was also studied in this work.

The results obtained are shown in Figure 20 and the kinetic and stoichiometric parameters in Table 7. Five pulses of feeding were supplied to the bioreactor and each pulse was added when an increase in DO was detected. Contrary to what was observed in the previous accumulation tests, VFAs were consumed to exhaustion in all the pulses. Substrate depletion was accompanied by an increase of DO and OUR decrease. Acetic acid was preferably consumed, followed by butyric and propionic acids. Despite no ammonium was added to the medium, the fact that the inoculum used was withdrawn from the SBR could explain the presence of some ammonium in the medium during the accumulation test.

Since ammonium is essential for growth, it can be assumed that most of the carbon consumed was redirected only to PHA production and cell maintenance. Because of this, growth rates were inferior than those obtained in the accumulation test without nutrient limitation. The maximum PHA content obtained was 34.6% of cell dry weight at 5.25 h, which was more 82.7% than at the beginning of the experiment. The overall yield $Y_{PHA/S}$ obtained in this accumulation test was 0.79 CmmolPHA/CmmolS. The produced copolymer had an average monomeric composition (on a molar basis) of 76:24 ± 1:1 (HB:HV), which coincided with the composition of the copolymer produced in the SBR at the same time. The monomeric composition of the copolymer was uniform throughout the test.

The PHA storage capacity in this accumulation test is much lower than others described in the literature. For example, Johnson et al. (2009) operated an acetate-fed SBR in order to select a PHA-accumulating MMC and evaluated the PHA storage capacity of the selected MMC in a fed-batch reactor under nitrogen limitation. The enriched mixed culture produced PHA up to a cellular content of 89 wt % within 7.6 h. Albuquerque et al. (2010b) also reached a maximum PHA content of 74.6% in a accumulation test, using as substrate a complex feedstock such as fermented sugar molasses.

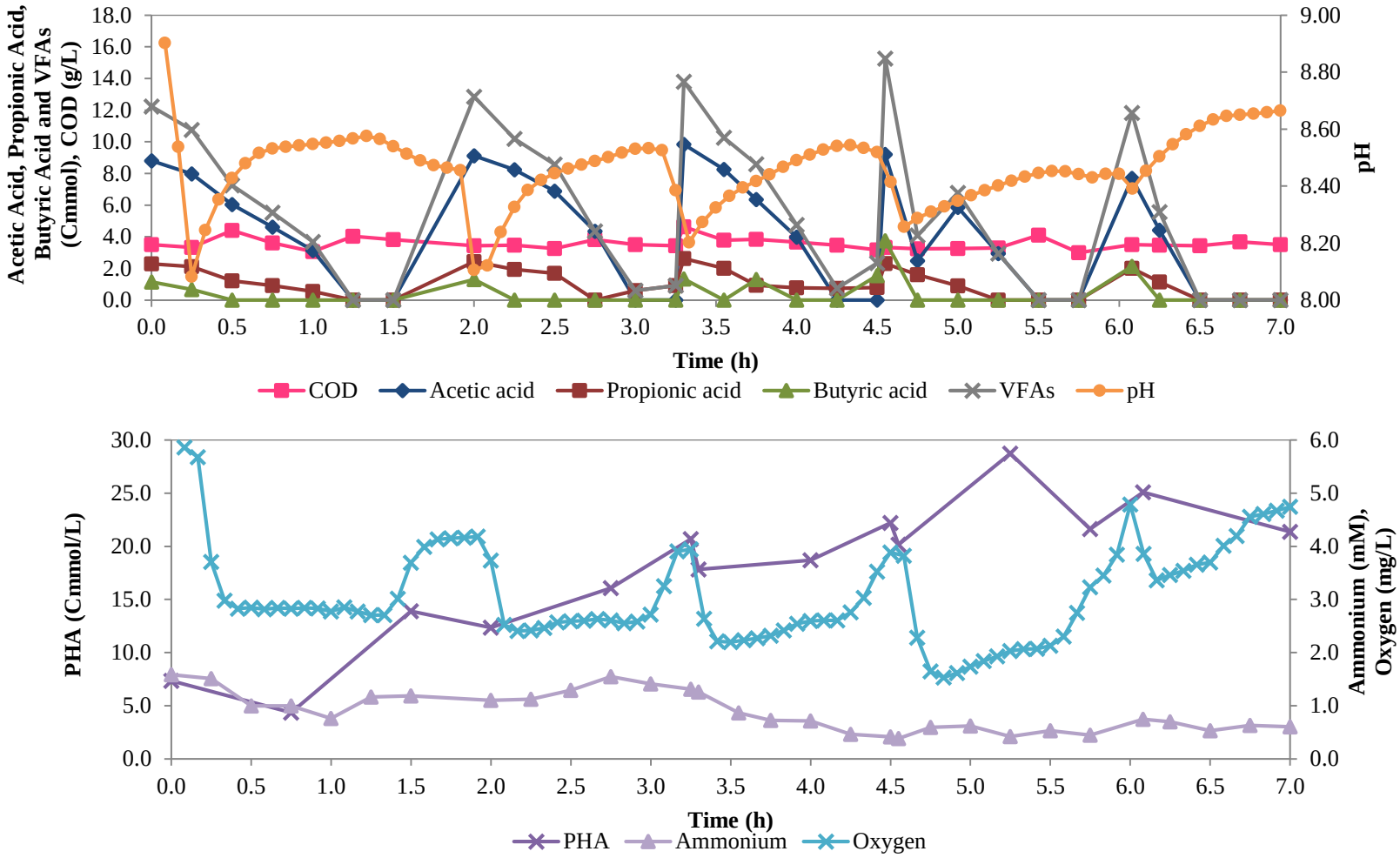


Figure 20. Evolution of pH, Oxygen, Ammonium, COD, PHA, Acetic Acid, Propionic Acid, Butyric Acid and VFAs concentrations in kinetic test performed with ammonium limitation. Test performed on the 68th day of operation

5.3. Microbial Community Characterization

5.3.1. Morphological Analysis

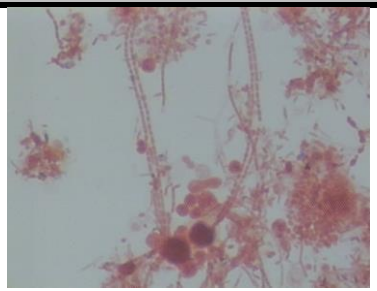
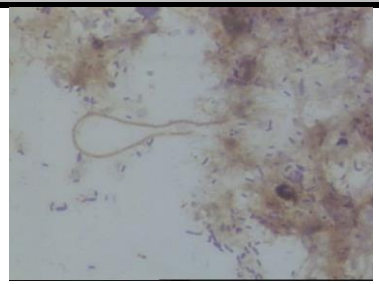
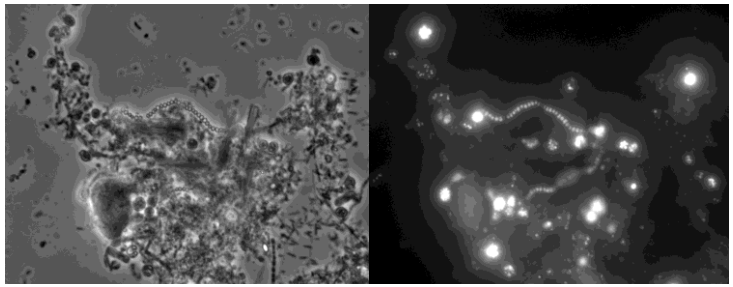
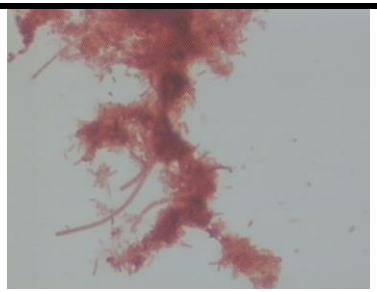
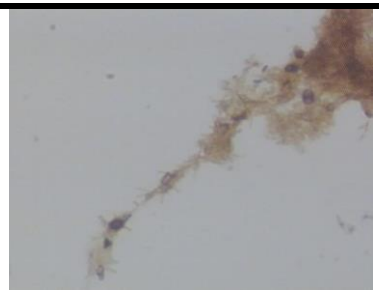
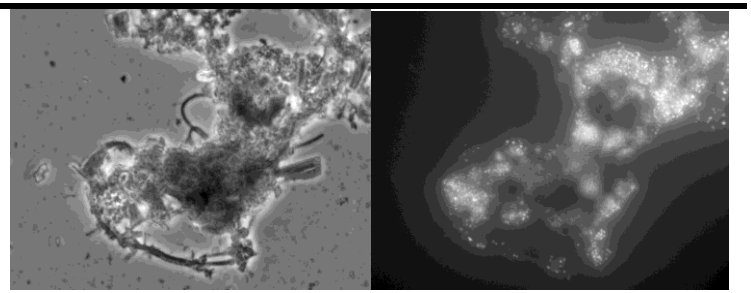
As stated previously, the analysis of the evolution of the microbial community throughout the reactor operation is crucial. The morphology of the selected MMC were analysed using several biological staining methods such as Gram, Neisser and Nile blue. Phase contrast microscopy was also used to identify the main morphotypes present in the microbial community.

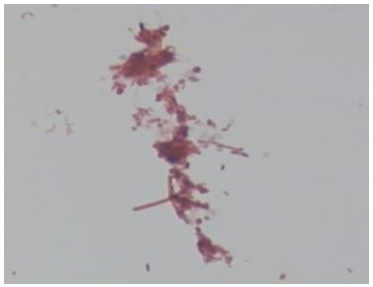
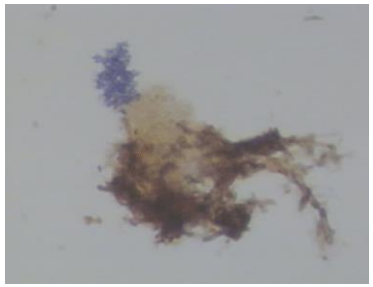
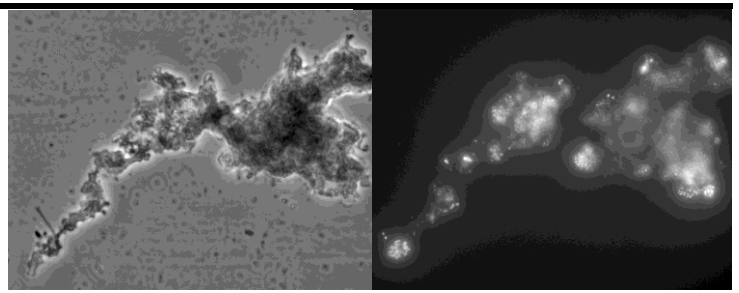
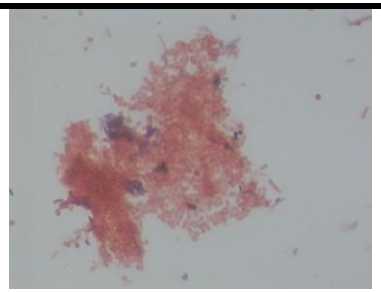
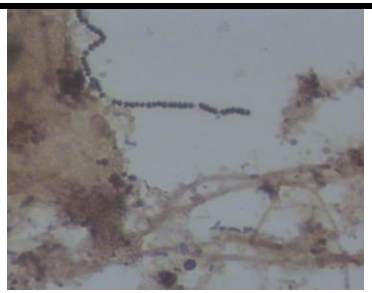
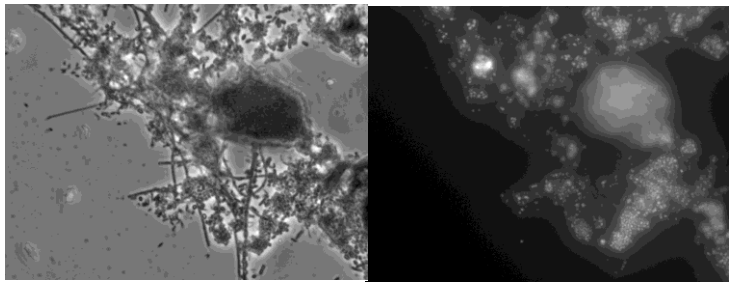
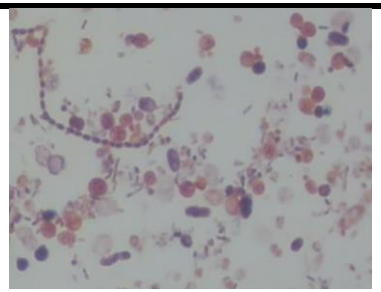
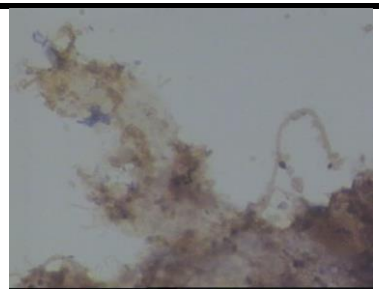
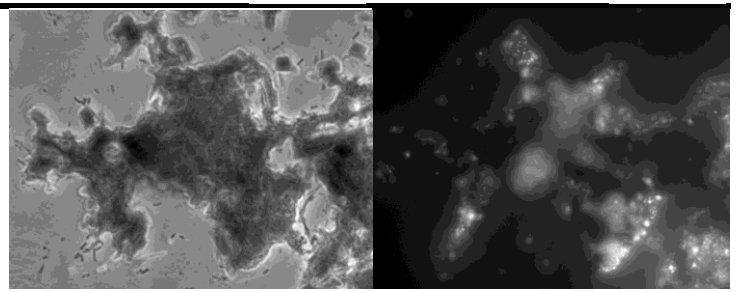
Gram, Neisser and Nile blue staining techniques were performed to original sludge and samples collected from the SBR on days 17, 32, 45, 51, 60 and 66 of operation. The results are summarized in Table 8. The MMC was dominated by Gram negative ($Gram^-$) cells throughout the entire operational period, yet a slight increase of Gram positive ($Gram^+$) cells was observed at the 55^{th} day of SBR operation. The high $Gram^-$ bacteria content is positive since these bacteria usually show good growth and higher PHA accumulation capability (Valappil et al 2006). With Gram staining it is also possible to infer about the cells' morphology. The dominant morphologies were *cocci* and *bacilli*, especially forming in dense aggregates. Some *streptobacilli* and *streptococci* were also detected.

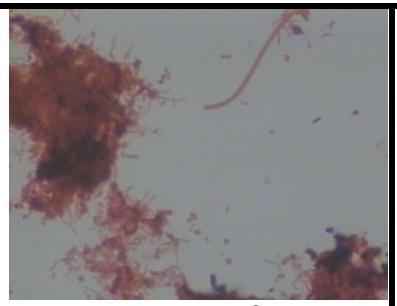
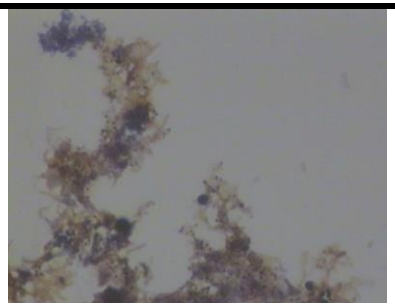
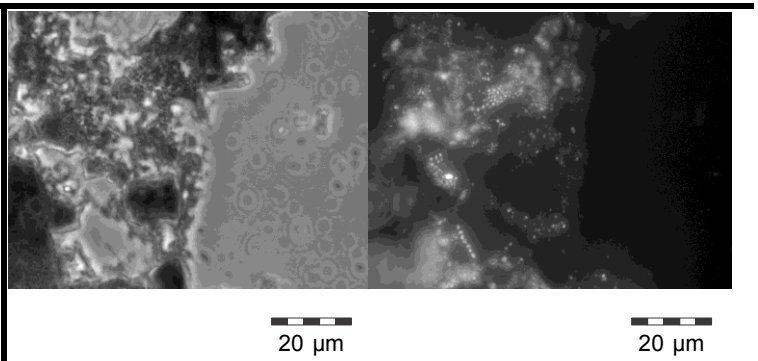
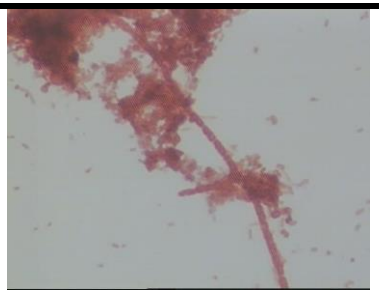
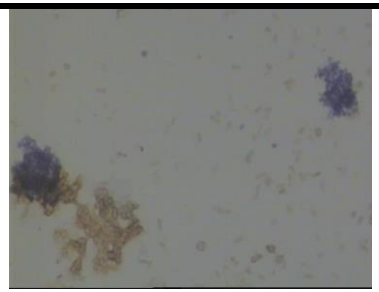
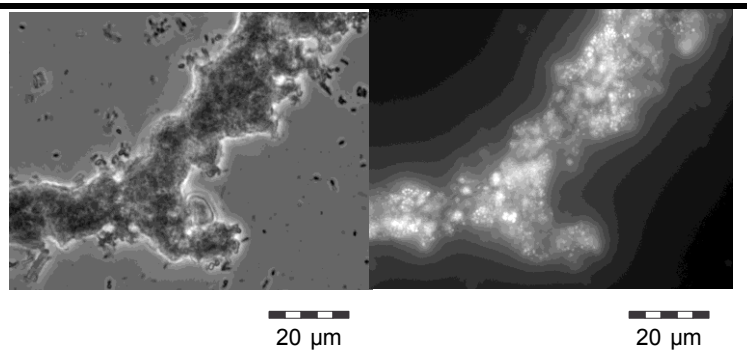
Regarding Neisser staining results, Neisser negative ($Neisser^-$) cells were dominant in the beginning of the operational period and an increase of Neisser positive ($Neisser^+$) cells was observed throughout the reactor operation. It is worth noting that some of the Neisser+ cells were totally stained instead of presenting dark spots on the edge of the cells, which are typical of the phosphorous accumulating organisms.

Nile Blue stain is a technique that can be used to confirm the presence of PHA-accumulating bacteria. Since the beginning of SBR operation, the presence of PHA inclusion bodies was easily noticeable by the existence of brilliant dots in the stained cells under epifluorescence.

Table 8. Micrographs of Gram, Neisser and Nile Blue stained samples collected from the SBR

Sample	Morphotypes	Gram Stain	Neisser Stain	Nile Blue stain (Phase contrast/Epifluorescence)
Original Sludge	*Cocci, bacilli, streptobacilli, streptococci.*	 Predominance of Gram$^-$	 Predominance of Neisser$^-$	
Day 17	Decrease in *streptobacilli* and *streptococci*. Dominance of *cocci* and *bacilli*.	 Predominance of Gram$^-$	 Predominance of Neisser$^-$	

Day 32	Dominance of *cocci* and *bacilli*.	 Predominance of Gram^{-}	 Increase of Neisser^{+}	
Day 45	Dominance of *cocci* and *bacilli*.	 Predominance of Gram^{-}	 Predominance of Neisser^{-}	
Day 51	Dominance of *cocci* and *bacilli*. Presence of big round *cocci* cells.	 Increase of Gram^{+}	 Predominance of Neisser^{-}	

Day 60	Dominance of *cocci* and *bacilli*. Disappearance of big round *cocci* cells, previously recorded.	 Predominance of Gram^{-}	 New increase in Neisser^{+} cells	
Day 66	Dominance of *cocci* and *bacilli*.	 Predominance of Gram^{-}	 Predominance of Neisser^{-} Aggregates of Neisser^{+} cells	

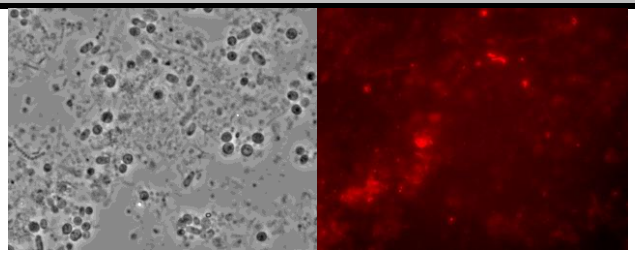
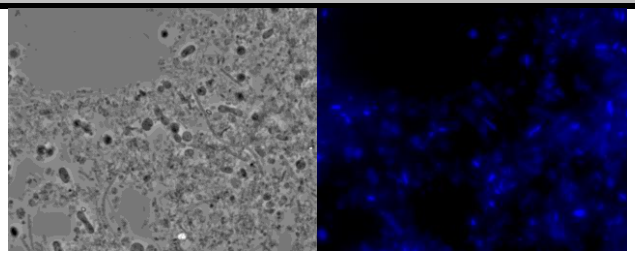
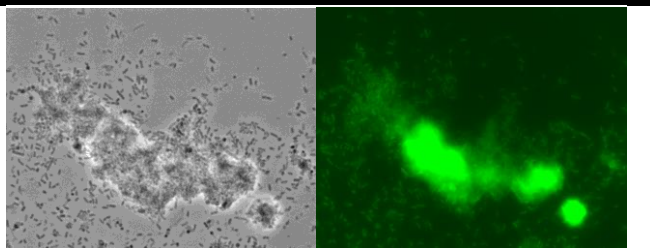
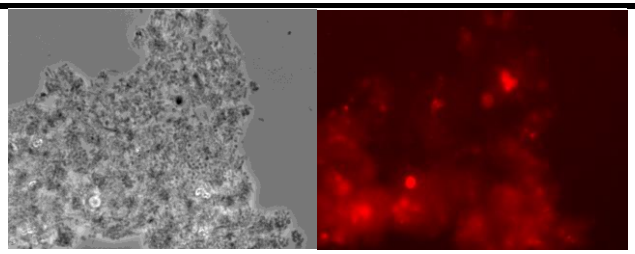
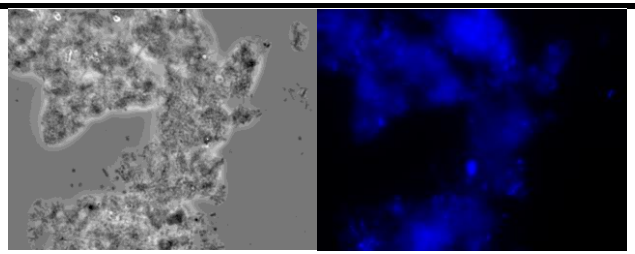
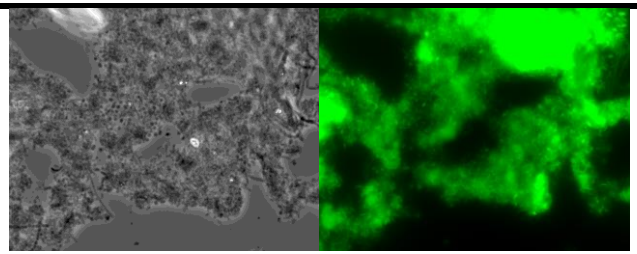
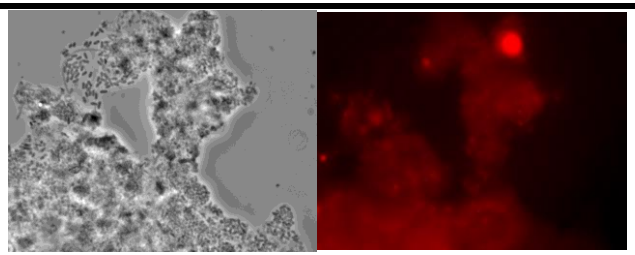
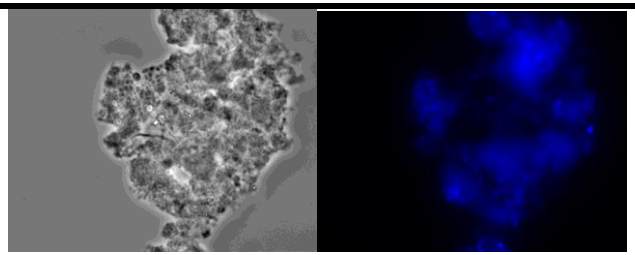
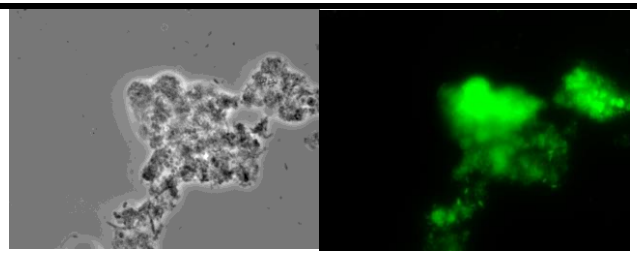
5.3.2. EPS Staining results

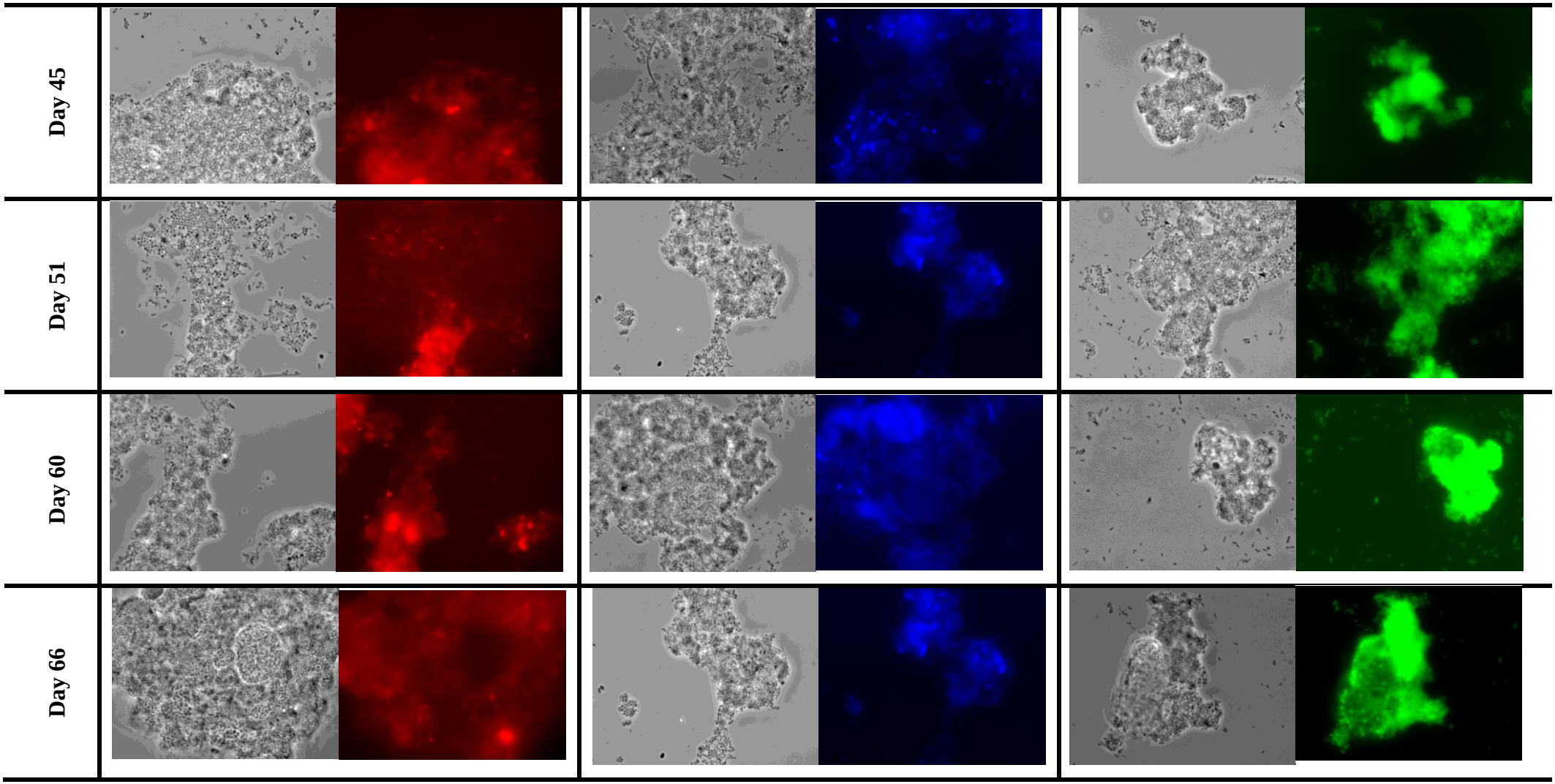
Because of the high tendency of the biomass to generate dense and indestructible aggregates, EPS production was evaluated using three different staining techniques specific for the different components of EPS. These techniques were performed to original sludge and samples collected from the SBR on days 17, 32, 45, 51, 60 and 66 of operation.

EPS is a highly hydrated gel that acts as a cementing substance in biofilms and flocs (de Beer et al 1996). Generally, EPS are understood to be extracellular polymers mainly composed of microbial polysaccharides. However, other extracellular polymeric substances may also be associated with EPS, such as proteins, nucleic acids and polymeric lipophilic compounds (Neu et al 2001). In this work, Calcofluor white was utilized to stain the β-D-glucopyranose polysaccharides, FITC was used to stain proteins and other amine-containing compounds and Concanavalin A was bonded to α-mannopyranosyl and α-glucopyranosyl sugar residues.

Table 9 shows the results of EPS staining. It is possible to see that the EPS content was inferior in the original sludge and on the 17^{th} day of operation and it increased throughout the reactor operation period. This leads to believe that EPS production could be related to the high toxicity of the HSSL, since EPS content suffered an increase throughout the reactor operation. The production of EPS can be an inconvenience since it requires consumption of substrate, deviating it from growth and PHA production, reducing the expected productivities.

Table 9. Pictures of the EPS staining results

Sample	α-glucans	β-glucans	Amino-sugars and proteins
Original Sludge			
Day 17			
Day 32			


Day 45
Day 51
Day 60
Day 66

5.3.3. FISH results

5.3.3.1. Microbial Community Identification

Throughout the SBR operating period, it is necessary to monitor the population in order to identify the different groups of microorganisms and relate them with the storage capacity and kinetics of the MMC (Queirós et al 2015). For these motives, FISH analysis was performed to original sludge and samples collected from the SBR on days 17, 32, 45, 51, 60 and 66 of operation.

In order to decrease the cellular aggregates of the MMC, which may negatively impact the visualization of FISH hybridised cells, a sludge pre-treatment using glass beads was performed. Since cellular aggregation still remained too high, an ultrasound treatment was applied, strongly reducing the size of microbial aggregates.

Initially, specific probes for the main Phyla within *Bacteria* domain were applied. The microbial community was mostly composed of *Bacteria* since no *Archaea* were detected. The ratio between *Bacteria* that hybridized with EUBmix probe and the total DAPI stained cells was over 70%, which is a normal result for FISH characterization of activated sludge samples. To continue the bacterial community identification, several FISH probes were applied taking into consideration *Bacteria* previously reported as PHA-accumulating organisms.

In order to identify the microbial community and to follow the MMC composition throughout the reactor operation, specific probes for the main groups within *Bacteria* domain were applied: *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, *Deltaproteobacteria, Chloroflexi, Flavobacteria, Bacteroidetes, Sphingobacteria*, *Actinobacteria*, *Firmicutes*, *Planctomycetales* (Table 5, section 3.7.6).

Figure 21 shows the evolution of the composition during the reactor operation. Since the beginning of the process, *Betaproteobacteria* were the dominant group and the relative abundance of *Betaproteobacteria* only suffered mild fluctuations during the whole reactor operation time. Since *Betaproteobacteria* were reported to possess PHA-producing ability, this shows that the operational conditions applied during the SBR operation favoured the maintenance of this group of bacteria within the MMC. *Alphaproteobacteria* were the second most dominant group in the bacterial community throughout the SBR operational time.

The bacteria community composition did not suffer major alterations throughout the whole operational period. *Flavobacteria*, *Bacteroidetes* and *Sphingobacteria* percentage suffered an increase on day 45 (from 4.96 ± 1.73% to 12.0 ± 1.77%) and *Actinobacteria* appeared in the culture on day 60 with a small percentage (0.65 ± 0.13%).

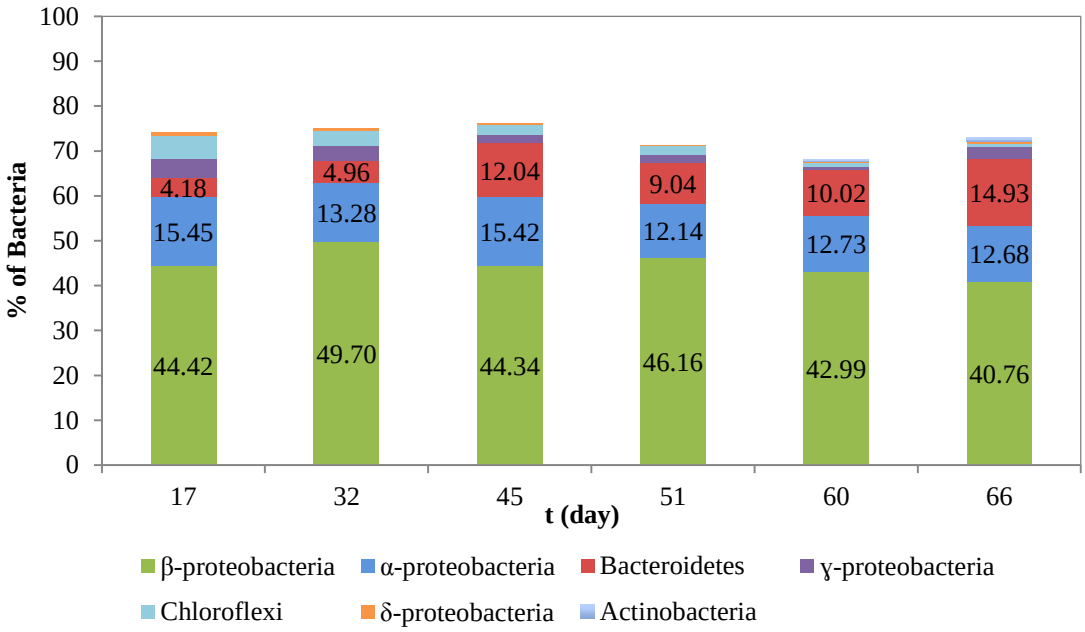


Figure 21. Bacterial community evolution throughout the SBR operation

Due to the high aggregation of the cells in the sample from the original sludge (inoculum), FISH quantification for this sample was not possible. However, the group-specific probes were also applied to this sample and after microscope observation of the results, it was possible to infer that *Betaproteobacteria* was the dominant group in the bacterial community, followed by *Alphaproteobacteria* and *Gammaproteobacteria.*

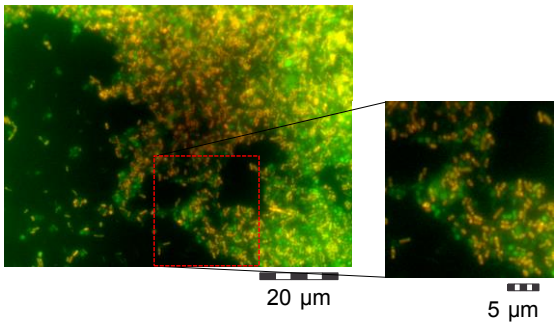
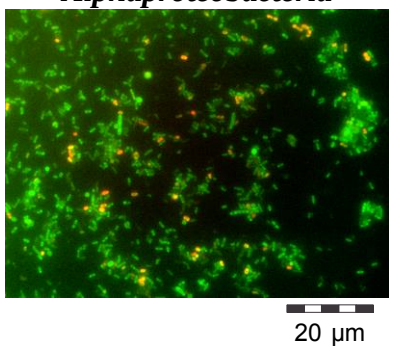
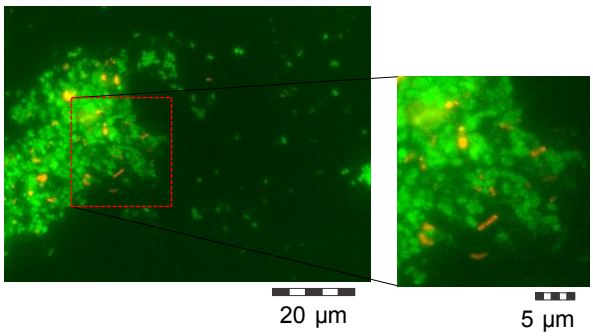
As previously mentioned, a stabilization of the culture occurred between the 60th and the 66th days and an apparent pseudo-stationary phase was established. By analysing the bacterial composition in these days, it is possible to conclude that the bacterial community did not suffer significant changes during this period. *Betaproteobacteria* remained as the dominant group and its relative abundance was constant during this period (42.99 ± 0.38% on day 60 and 40.76 ± 2.15% on day 66).

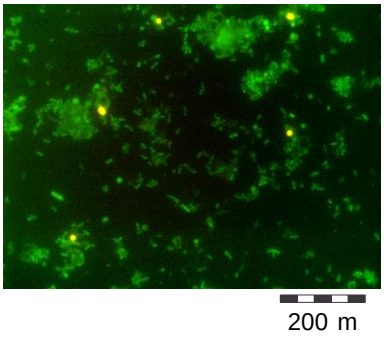
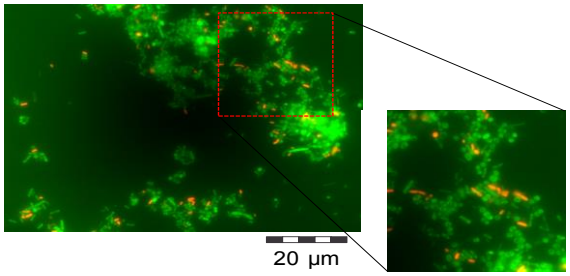
Previous studies from Albuquerque et al. (2013), Lemos et al. (2008), Moita and Lemos (2012), Queirós et al. (2014) and Ferreira et al. (2015) have identified organisms

belonging to *Alpha-*, *Beta-* or *Gammaproteobacteria* classes as PHA-accumulating organisms selected under ADF. Results obtained in this study are in line with those described in the literature.

Table 10 summarizes the main identified morphotypes belonging to different groups within Bacteria domain.

Table 10. Bacterial groups identified

Bacterial group	Main morphotypes	Morphology description
Betaproteobacteria 	*Bacilli* and *cocci*	✓ Mostly large *bacilli* cells with visible PHA granules; ✓ Nile blue results showed that this morphotype was the principal PHA producer.
Alphaproteobacteria 	*Bacilli* and *cocci*	✓ Mostly *cocci* and some *bacilli* cells; ✓ Large *cocci* cells.
Gammaproteobacteria 	*Bacilli* and *cocci*	✓ Mostly *cocci*; ✓ Large *cocci* cells; ✓ Long *bacilli cells.*

Deltaproteobacteria 	*Cocci*	✓ Large and round *cocci* cells.
Flavobacteria, Bacteroidetes* and *Sphingobacteria 	*Bacilli and cocci*	✓ Small *bacilli* and *cocci* cells.

5.3.3.2. Analysis at genus level

Bacterial community identification continued using specific probes for genera inside *Beta-* and *Alphaproteobacteria*, since these were the dominant groups present in the culture. This evaluation was only performed for samples belonging to the pseudo-stationary phase (on 60th and 66th days).

Regarding the *Alphaproteobacteria* group, specific probes for *Sphingomonas* (Beer et al 2004), *Defluvicoccus* (Meyer et al 2006) and *Defluvicoccus* related Tetrad Forming Organism (TFO-DF) (Wong et al 2004) were applied. Positive results were only obtained for *Defluvicoccus* related TFO-DF (0.78 ± 0.38%), as shown in Figure 22.

A specific probe for *Amaricoccus* was also tested, since this genus was reported as having PHA-storing ability (Lemos et al 2008). However, no positive binding of the specific probe was found.

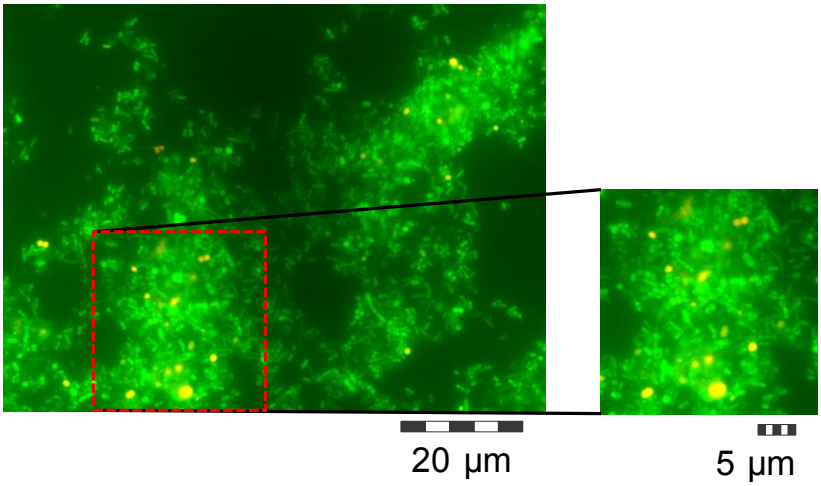


Figure 22. FISH picture of TFO-DF on day 60. Green cells are hybridized with EUBmix and the yellow cells are hybridized with TFO-DF 218+618 probe

For the *Betaproteobacteria* group, specific probes for *Thauera* and *Azoarcus* genera were applied due to their previous identification as PHA-accumulating organisms (Dionisi et al 2005a; Dionisi et al 2005c; Serafim et al 2006; Albuquerque et al 2013). Positive results were obtained for both genera as can be seen in Figure 23 and Figure 24. However, only small amounts of *Thauera* (0.72 ± 0.25%) and *Azoarcus* (0.62 ± 0.02%) were found.

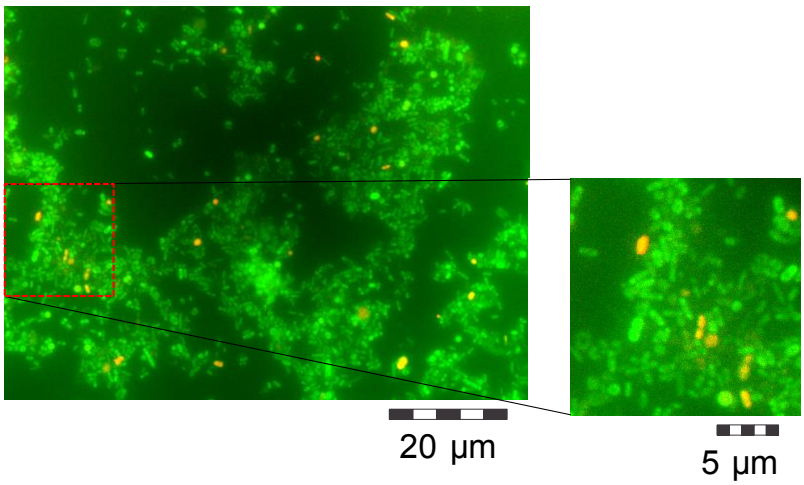


Figure 23. FISH picture of *Thaurea* on day 66. Green cells are hybridized with EUBmix and the yellow/red cells are hybridized with THAU646 probe

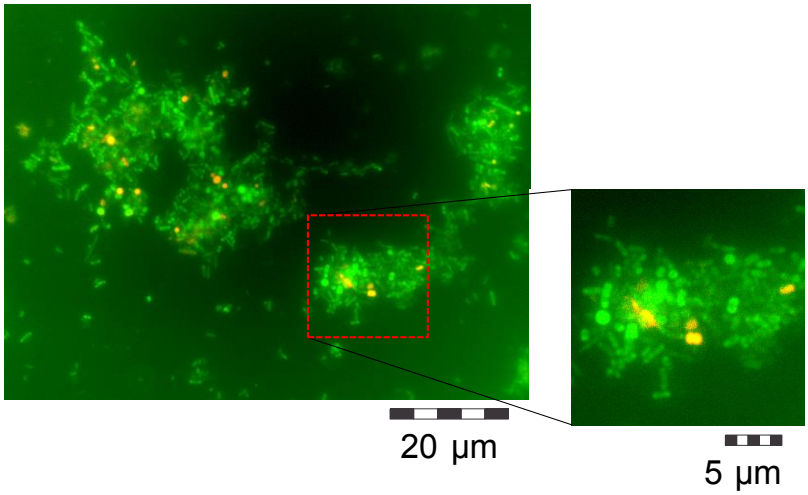


Figure 24. FISH picture of *Azoarcus* on day 60. Green cells are hybridized with EUBmix and the yellow cells are hybridized with AZO644 probe

After applying available genera specific probes for the *Betaproteobacteria* group, the main part of this population remained unidentified. The identification of *Betaproteobacteria* by FISH analysis was only possible once the sequence data from 16S rRNA gene clone library was available. During the analysis of the sequence data, 16S rRNA gene sequences belonging to the *Acidovorax* genus appeared quite frequently. Moreover, *Acidovorax* was previously shown to be capable of PHA storage (Schulze et al 1999). Schulze et al. (1999) developed the probe ACI145 based on 16S rRNA gene sequences of the available *Acidovorax* species. The probe ACI145 with the sequence 5'-TTTCGCTTCGTTATCCCC-3', was synthetized by BioFab (Rome, Italy) and applied to all samples. Positive results were obtained for all the screened samples and it was observed that *Acidovorax* content in the microbial community increased from 2.62 ± 0.93% (in day 17) to 28.98 ± 2.51% (in day 66). During the pseudo-stationary phase, the *Acidovorax* genus became the major constituent of the *Betaproteobacteria*, constituting 71% of the *Betaproteobacteria* population on day 66 (Figure 25). The results of the probe hybridization are shown in Figure 26.

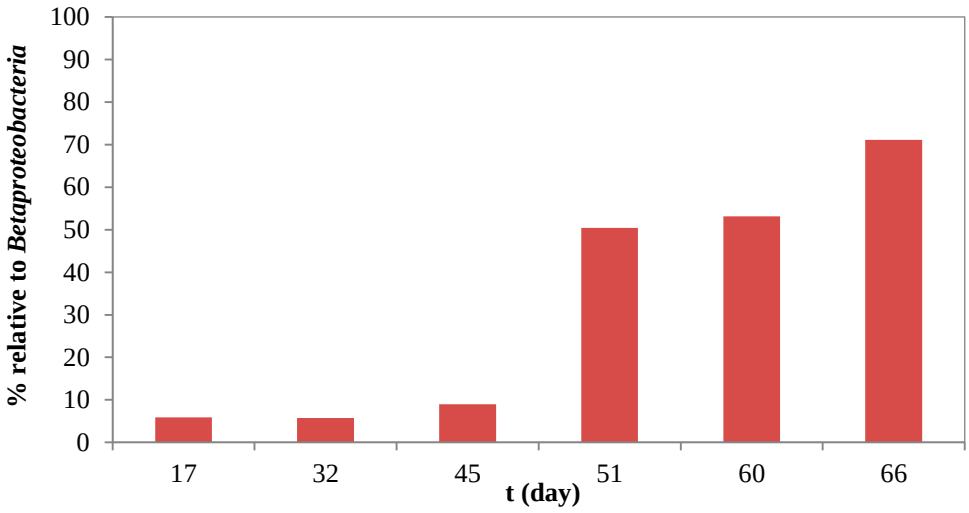


Figure 25. *Acidovorax* content evolution throughout the reactor operation. Percentages are relative to the relative abundance of *Betaproteobacteria*

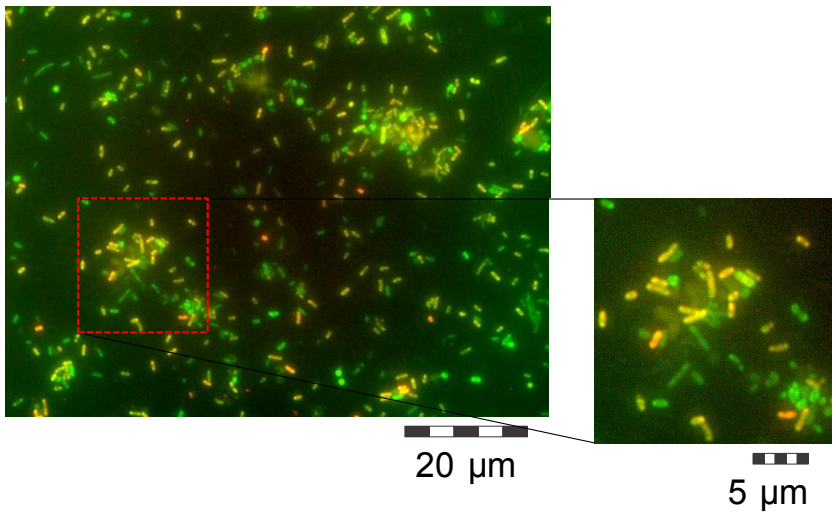


Figure 26. FISH picture of *Acidovorax* on day 66. Green cells are hybridized with EUBmix and the yellow cells are hybridized with ACI145 probe

5.3.4. 16S rRNA gene Clone Library Results

To identify the main bacteria responsible for PHA accumulation in the selected MMC, a 16S rDNA clone library was constructed. The sample used to extract the DNA was collected from the SBR on the 66th day of operation. A total of 108 white colonies were screened, and after performing PCR and electrophoresis to check the occurrence of amplification, 60 clones were chosen for partial sequencing. The obtained 16S rDNA partial sequences were then analysed by BLAST and grouped by genera (Table 11).

The major part of the clones present in the clone library belonged to *Betaproteobacteria* class. Inside this class, genera *Acidovorax* and *Simplicispira* were the most represented, but *Rhodoferax, Comamonas, Ramlibacter, Hydrogenophaga, Limnohabitans, Giesbergeria, Albidiferax, Thauera,* and *Variovorax* were also detected.

The second most dominant class was *Alphaproteobacteria*, including genera *Paracoccus, Agrobacterium* and *Shinella* as the most represented and *Catellibacterium* and *Novosphingobium* in minor amounts. These results were also in line with the FISH analysis.

Regarding the less abundant genera in the clone library, the genus *Pseudoxanthomonas,* belonging to the *Gammaproteobacteria* group, and genera *Leadbetterella* (*Cytophagia* group) and *Salinirepens* (*Flavobacteria* group) were also recovered in the clone library.

These results were expected since, during the pseudo-stationary phase, *Betaproteobacteria* was the dominant group of the bacterial community (40.76 ± 2.15%) followed by *Alfaproteobacteria* (12.68 ± 0.79 %) and *Gammaproteobacteria* was only present at a small percentage (2.55 ± 0.54).

Some species of *Acidovorax* (Schulze et al 1999), *Thauera* (Dionisi et al 2005c), *Comamonas* (Saito and Doi 1994), *Paracoccus* (Ueda et al 1992) and *Hydrogenophaga* (Choi et al 1999) genera were already described as PHA-accumulating organisms.

Ferreira et al. (2015) isolated and characterized organisms able to store PHA, from a MMC selected under ADF conditions. A16S rRNA gene clonal analysis was performed and clones related to *Comamonas* spp. and *Novosphingobium* spp. were also detected. However, in the work of Ferreira et al. (2015), the MMC selection was performed using unfermented HSSL, which can explain the differences in the microbial community composition.

Table 11. Clone library results

Microorganism	Sequence name	Clone number	% of similarity between sequences	Affiliation at group level
Acidovorax	Catarina_0	1	≥ 97%	*Betaproteobacteria*
	Catarina_0	5		
	Catarina_0	21		
	Catarina_1	39		
	Catarina_1	41		
	Catarina_1	46		
	Catarina_1	51		
	Catarina_2	85		
	Catarina_3	87		
	Catarina_3	92		
	Catarina_3	102		
	Catarina_3	104		
	Catarina_3	107		
Acidovorax "like"	Catarina_4	126	94 - 96%	
	Catarina_5	132		
	Catarina_5	141		
	Catarina_5	145		
	Catarina_6	149		
	Catarina_6	155		
	Catarina_6	157		
Comamonas	Catarina_0	2	100%	*Betaproteobacteria*
	Catarina_2	75		
Leadbetterella	Catarina_0	12	94%	*Cytophagia*
	Catarina_3	105		
Catellibacterium	Catarina_0	39		*Alphaproteobacteria*
Ramlibacter	Catarina_1	40	96%	*Betaproteobacteria*
	Catarina_1	43		
Giesbergeria	Catarina_1	57		*Betaproteobacteria*
Salinirepens	Catarina_1	60		*Flavobacteria*
Paracoccus "like"	Catarina_2	66	92 - 96%	*Alphaproteobacteria*
	Catarina_2	72		
	Catarina_3	90		
Paracoccus	Catarina_3	100	≥ 98%	
	Catarina_4	113		
	Catarina_6	158		
Agrobacterium	Catarina_2	67	100%	*Alphaproteobacteria*
	Catarina_4	117		
Rhodoferax	Catarina_2	68	≥ 99%	*Betaproteobacteria*
	Catarina_2	86		
	Catarina_5	83		
Simplicispira	Catarina_2	70	≥ 97%	*Betaproteobacteria*
	Catarina_2	76		
	Catarina_4	118		
	Catarina_5	133		
	Catarina_5	136		
	Catarina_5	147		
	Catarina_6	148		
	Catarina_6	164		
Shinella	Catarina_3	101	99%	*Alphaproteobacteria*
	Catarina_5	139		
Novosphingobium	Catarina_4	108		*Alphaproteobacteria*
Hydrogenophaga	Catarina_4	123	100%	*Betaproteobacteria*
	Catarina_4	125		
Albidiferax	Catarina_4	131		*Betaproteobacteria*

Thauera	Catarina_5	134		*Betaproteobacteria*
Pseudoxanthomonas	Catarina_5	146		*Gammaproteobacteria*
Limnohabitans	Catarina_6	152	98%	*Betaproteobacteria*
	Catarina_6	154		
Variovorax	Catarina_6	163		*Betaproteobacteria*

After analysing the results of the partial sequencing, 15 clones were chosen for complete sequencing and refinement of their taxonomic affiliation. 16S rDNA sequences were manually aligned and analysed by BLAST and the highest similarity was determined, Table 12. The obtained sequences were then submitted to GenBank.

Several microorganisms present in the clone library were already described as PHA-accumulating organisms in pure culture: *Comamonas acidovorans* using 1,4-butanediol or 4-hydroxybutyric acid, methanol and *n*-amyl alcohol as substrate (Saito and Doi 1994), *Paracoccus denitrificans* consuming γ-butyrolactone alone or with fructose or butyric acid (Ueda et al 1992), *Alcaligenes eutrophus* using soya wastes from a soya milk dairy (Doi et al 1990) and *Alcaligenes latus* using malt wastes from a beer brewery plant as substrate (Yu et al 1999). *Acidovorax* spp. (Schulze et al 1999) was also described as a PHA producer.

Table 12. Results of the complete sequencing

Sequence name	Clone number	Highest Similarity	% Identity	GenBank Acession number
CR1	2	*Acidovorax caeni*	94%	KT262954
CR2	20	*Acidovorax wautersii*	98%	KT262955
CR3	60	*Fluviicola taffensis*	92%	KT262951
CR4	105	*Leadbetterella byssophila*	95%	KT262956
CR5	108	*Comamonas testosteroni*	90%	KT262952
CR6	113	*Paracoccus siganidrum*	98%	KT262957
CR7	117	*Agrobacterium tumefaciens*	99%	KT262958
CR8	118	*Acidovorax radicis*	98%	KT262959
CR9	124	*Simplicispira metamorpha*	98%	KT262960
CR10	125	*Comamonas testosteroni*	99%	KT262961
CR11	128	*Alcaligenes aquatilis*	94%	KT262962
CR12	139	*Shinella zoogloeoides*	99%	KT262953
CR13	146	*Pseudoxanthomonas kaohsiungensis*	99%	KT262963
CR14	163	*Acidovorax delafieldii*	98%	KT262964
CR15	164	*Simplicispira metamorpha*	97%	KT262965

A tree showing the phylogenetic diversity among the 15 clones is shown in Figure 27. The evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993).

All the sequences were related to each other and to PHA-producing microorganisms already described in the literature and previously found in culture selection processes for PHA production. Six (CR1, CR2, CR8, CR9, CR14 and CR15) of the 15 sequences were related to *Acidovorax*. Clones CR1, CR2 and CR15 were closely related to *Acidovorax caeni* and clones CR8 and CR14 to *Acidovorax radicis.* Contrarily to what was revealed by the BLAST search, the clone CR9 was closer to *Acidovorax wautersii* than to *Simplicispira metamorpha*. Clone CR15 was also closer to *Acidovorax caeni* than to *Simplicispira metamorpha*. Two sequences (CR5 and CR10) were related to *Comamonas testosteroni*. Sequences CR3, CR4, CR6, CR7, CR11, CR12, and CR13 were related to *Fluviicola taffensis, Leadbetterella byssophila, Paracoccus siganidrum, Agrobacterium tumefaciens, Alcaligenes aquatilis*, *Shinella zoogloeoides* and *Pseudoxanthomonas kaohsiungensis*, respectively.

Lampropedia hyalina (Beccari et al 2010; Valentino et al 2014), *Azoarcus sp*. (Serafim et al 2006), *Thauera sp*. (Dionisi et al 2005c), *Bacillus megaterium* (Omar et al 2001), *Plasticicumulans acidivorans* (Valentino et al 2014), *Thiocystis violacea* (Sudesh et al 2000) and *Zoogloea sp.* (Moita and Lemos 2012) are microorganisms with phylogenetic relationships with the clones and are known PHA producers previously found in MMC selection processes for PHA production. Moreover, *Alcaligenes* sp. (Doi et al 1990; Yu et al 1999) is a well known PHA producer. *Comamonas testosteroni* (Kasuya et al 1994) and *Acidovorax facilis* (Mergaert et al 1993) were already described as PHA degrading microorganisms. Once again, these results prove that the culture selection process was successful.

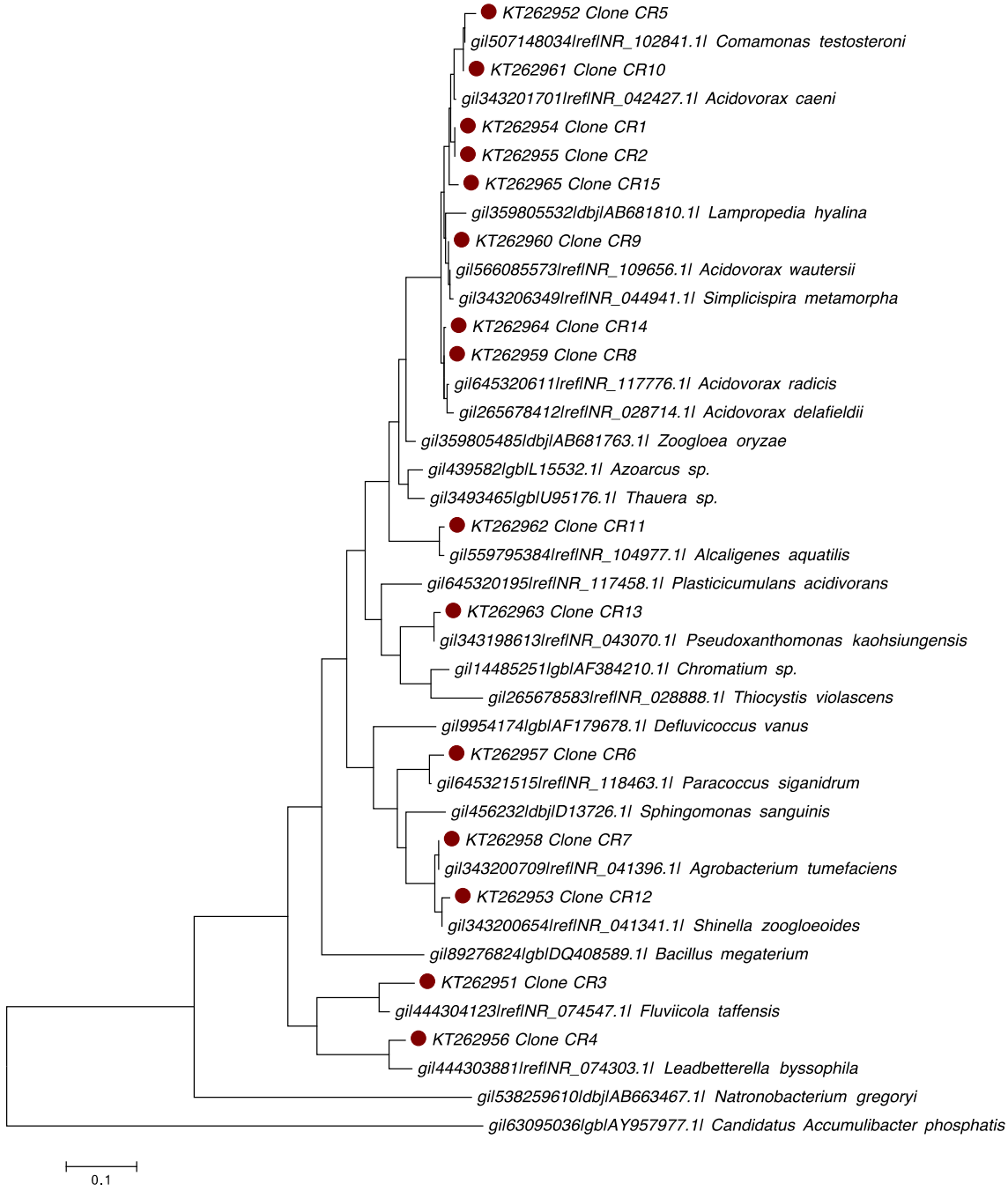


Figure 27. Phylogenetic tree based on full-length nucleotide sequences of 16SrRNA gene of clones CR1-15 (in the tree preceded by a filled red dot). Molecular Phylogenetic analysis by Maximum Likelihood method. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 42 nucleotide sequences. Evolutionary analyses were conducted in MEGA6 (Tamura et al 2013)

6. Concluding remarks

In this work, a three-step process was used for PHA production. In the first step, HSSL, a by-product of pulp industry, was submitted to acidogenic fermentation in order to produce a mixture rich in VFAs, which was used as substrate in the next two steps. In the second step, a SBR was operated for 70 days under ADF conditions in order to select a MMC able to produce PHA. A pseudo-stationary state was reached after 60 days of SBR operation, which indicated that the MMC was able to successfully adapt to the carbon source. In the third step, the ability of the selected MMC to accumulate PHA was tested. Three accumulation tests were performed and the selected MMC reached a maximum PHA content of 47.1%.

FISH analysis showed that *Betaproteobacteria* was the dominant phylogenetic group in the bacterial community. On day 66, *Betaproteobacteria* constituted 40.8 ± 2.2% of total bacteria. The remaining bacteria belonged mostly to the *Alphaproteobacteria* (12.7 ± 0.8%), *Gammaproteobacteria* (2.55 ± 0.5%) and *Bacteroidetes* (14.9 ± 1.7%) groups. Inside *Betaproteobacteria*, a considerable amount of *Acidovorax* (28.9 ± 3.1%) was detected. The typical genera found in MMCs selected in SBRs operated under ADF conditions, *Thauera* and *Azoarcus*, were only found in small amounts.

Nile Blue results revealed the high abundance of PHA inclusion bodies that were maintained since the beginning of SBR operational period.

Clone library results presented several clones that were already described as PHA-producers. These results also matched with the FISH analysis, since the major part of the clones belonged to the *Betaproteobacteria* group. Moreover, a significant number of clones belonged to the *Acidovorax* genera, which was already described as a PHA-producer. Phylogenetic analysis proved that the clones were related with PHA-producing organisms.

The results obtained in this work showcase the potential of the three-step process for PHA production. With 70 days of SBR operation, a culture with high abundance of PHA producing organisms was selected, which indicates that the selection process was well performed.

7. Future prospects

As already mentioned the MMC response during the selection process is influenced by different factors. For this motive, this research should focus on the influence of the reactor operating conditions in the MMC selection process. Some of the operational conditions to be studied are: SRT, HRT, pH, temperature, cycle length, OLR, influent substrate concentration and nutrient concentration. PHA production under phosphorus and ammonium limitation should also be the target of further investigation.

It is known that the VFA mixture made available to the PHA-storing microorganisms will influence the PHA composition and HB:HV ratio, which in turn affects PHA physical and mechanical properties. Testing different operational conditions during the acidogenic fermentation step to determine their influence in VFA production and in polymer composition should also be considered.

Throughout the SBR operational time, *Acidovorax* content suffered an increase. Since this organism is a known PHA accumulator, a bioaugmentation strategy can be adopted in order to promote the *Acidovorax* growth inside the bacterial community. This study should focus on factors such as *Acidovorax* growth conditions, VFA preference and possible changes in the operational conditions.

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